In Vitro Investigation of Tooth Erosion

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Abstract

In vitro investigations in dental research are important as they allow for conditions such as tooth erosion to be extensively studied in controlled environments for product development and testing. The studies in this thesis investigate citric, phosphoric and hydrochloric acids under varying conditions of concentrations and immersion times in erosion and erosion-abrasion models and use non-contact profilometry and Knoop microhardness to measure the change in the surface of enamel. The effects of different experimental protocols on profilometry and Knoop microhardness were investigated. Using the results from these preliminary studies, a modified erosion model was developed to investigate the effects of low concentration fluorides and time of application. The role of fluoride experiment was furthered by investigating a dose response effect using sodium and stannous fluoride at concentrations normally found in mouth rinses and toothpastes.

Citric and phosphoric acid were more erosive than hydrochloric acid at pH 3.2. The effect of increasing the immersion time and concentration increased the amount of erosion. The addition of abrasion produced a non-linear response, suggesting a more complex mechanism was operating rather than the simple eroded surface being more susceptible to abrasion. Profilometry and to a lesser extent Knoop microhardness were effective measurements to quantify the amount of erosion. Tooth surface/type, ultrasonication, storage, agitation and speed, rinsing, volume and position of sample all influenced the mean step height and Knoop microhardness change. Stannous fluoride (225ppm) produced significantly lower ($p<0.001$) mean step height and higher Knoop microhardness change than sodium fluoride. The application before an erosive challenge produced a significantly lower mean step height ($p<0.04$) for stannous fluoride compared to the application after. A dose response effect was observed between the different fluorides. Both fluorides produced significantly lower mean step height ($p<0.001$) and Knoop microhardness ($p<0.001$) change compared to the control.

Sodium fluoride provided less protection (significantly higher mean step height) ($p<0.05$) compared to stannous fluoride. These studies show that the different experimental protocols can influence the measured outcome and that further work is needed to fully understand the effects of all the experimental protocols and abrasion. Greater standardisation and detailed reporting in method sections need to be promoted in *in vitro* dental research.

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Preface

There are nine chapters in this thesis. The first chapter is a critical review of the literature. The second chapter describes the materials and methods that were common to all experiments in this thesis and the measurement techniques used.

Chapters three, four, five and six are the experimental chapters and all follow a similar layout. Each chapter has an introduction followed by the aim, objectives and hypotheses. The materials and methods specific to that chapter and its experiments are then presented followed by the results, discussion and then a summary.

Chapter three investigates the effect of different acids, immersion times, concentrations and abrasion on *in vitro* erosive tooth wear.

Chapter four investigates the effect of the different model variables that are used when conducting *in vitro* erosion to see how they affect the tooth surface loss and Knoop microhardness change.

Chapter five uses the knowledge gained from the previous two chapters to appropriately modify the protocol to study the effects of low concentration stannous and sodium fluoride solutions on erosion and whether applying before or after an erosive challenge affects the efficacy.

Chapter six expands on Chapter five by including more robust quality control measures for sample preparation and investigating a dose response effect of sodium and stannous fluoride at 0, 50, 225, 450 and 1450ppm on an *in vitro* erosion model.

Chapter seven is a general discussion for the whole thesis and includes final conclusion and suggestions for the future work.

Chapters eight and nine are the references and appendices respectively.

Declarations

All statistical analysis in this thesis was performed by Manoharan Andiappan.

As there were no positive step height values in the data, the terms ‘step height’ or ‘mean step height’ refer to step height loss.

As all the Knoop microhardness measurements on the worn enamel were softer than the reference surface and the change was calculated by subtracting the worn hardness value away from the reference hardness; ‘Knoop microhardness change’ and ‘mean Knoop microhardness change’ refer to a loss of hardness.

Abbreviations

MSH: Mean step height

SLMSH: Single line mid-point step height

KHC: Knoop microhardness change

NaF: Sodium Fluoride

SnF_2: Stannous Fluoride

TiF_4: Titanium Tetra Fluoride

AFM: Atomic force microscopy

NaOH: Sodium Hydroxide

AmF: Amine Fluoride

FEPA: Federation of European Producers of Abrasives

EA: Erosion-Abrasion

SD: Standard Deviation

TMR: Transverse microradiography

SEM: Scanning electron microscopy

SEM-EDX: Scanning electron microscopy: Energy-dispersive X-ray spectroscopy

AAS: Atomic Absorption Spectroscopy

DOM: Demineralised Organic Matrix

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Chapter 1. Literature Review

1.1 Tooth wear

Tooth wear is described as a multifactorial process that includes erosion, abrasion and attrition potentially leading to the irreversible loss of enamel and dentine (Bartlett & Smith 2000). Erosion is defined as non-bacterial tooth surface loss from chemical or electrolytic mechanisms by extrinsic or intrinsic acids acting on plaque free tooth surfaces. Abrasion is defined by the physical wear resulting from the mechanical action of foreign substances and attrition is defined as the wear by the action of antagonistic teeth (Ganss 2006; Imfeld 1996).

1.2 Erosion

Erosion, acid erosion and dental erosion define a similar process, but for the thesis, the term erosion will be adopted. The acids that cause erosion can originate from intrinsic or extrinsic sources. Erosion from intrinsic acids is caused by gastric contents reaching the oral cavity and is associated with vomiting, regurgitation, gastro-oesophageal reflux or rumination (Scheutzel 1996). Pure gastric acids have a pH of approximately 1-2 (Bartlett & Smith 1994) and frequent exposure will cause erosion. The factors affecting erosion from extrinsic acids can be environmental (occupational), diet, acidic medication and lifestyle (behavioural) (Zero et al. 2000).

The main dietary acids that are associated with erosion are citric, phosphoric, malic and tartaric acids. Fruit, acidic candies, soft drinks, fruit-based drinks and wine contain citric and malic acid (West et al. 2000). Soft drinks can also contain phosphoric, ascorbic, lactic and carbonic acid. However the erosive potential of carbonic acid may not be important in erosion and this means carbonated water is not erosive (Parry et al. 2001). Wine also contains tartaric acid with small amounts of succinic acid.

1.3 Abrasion

Sources of abrasion arise from the consumption of food, oral hygiene products, holding or unusual rubbing of foreign objects on enamel (hair grips, pipe smoking, screws/nails) and incorrect or excessive pressure when brushing teeth (Pickles 2006; Kelleher & Bishop 1999; Mair 1992; Addy & Shellis 2006). Pure abrasion is unlikely to cause clinically significant tooth wear because direct contact must be made with materials that are equal to, or harder than enamel. Harder substances plastically deform the enamel producing 'grooves' which progress to become 'shoulders' and then cracking of the shoulders leads to the loss in tooth height (Pickles 2006). Tooth brushing is the most commonly reported source of abrasion, however, when used alone, it causes a negligible amount of tooth wear (Voronets & Lussi 2010). It is only in combination with erosion that abrasion causes a greater risk to enamel (Ganss 2006).

1.4 Attrition

Tooth- to-tooth wear is termed attrition. It seems to behave quite differently from other tooth wear processes such as erosion and abrasion. The additional factor influencing the severity of wear is the action of the muscles of mastication. The combination of attriting teeth and bruxism seems to have a different impact on teeth causing a more mechanical type wear (Bartlett & Smith 2000). Although an important contribution to tooth wear it will not be reviewed in depth in this thesis.

1.5 Abfraction

This is a purely theoretical concept and has not been shown to occur clinically. The theory is based around the vertical movement of the tooth in the periodontal ligament and the formation of stress areas around the cervical margin (Lee & Eakle 1984). The concept has been supported by some laboratory evidence but lacks any clinical investigations. A critical review assessed the contribution from abfraction and indicated that there is insufficient evidence to support its existence at the present time (Sarode & Sarode 2013; Bartlett & Shah 2006).

1.6 Current concepts on the early erosive lesion

Erosion can be irreversible. Early stages involve the diffusion of acid onto enamel causing demineralisation and softening but no bulk tissue loss (Laurance-Young et al. 2011) and this allows the possibility of remineralisation by saliva or a dental product (Ten Cate 2000; Featherstone & Lussi 2006; Lussi et al. 2011). Erosion is understood to initially soften the enamel surface and the depth of the softening depends on the strength of the erosive solution. The softening has been estimated to penetrate between 0.2 and 5μm into the bulk of enamel (Voronets & Lussi 2010). This superficial, demineralised and softened layer is more easily removed by mechanical action such as abrasion if there is insufficient time to remineralise and harden the surface. Provided no mechanical action occurs the current opinion is that the surface can be remineralised without any change in the surface. However, there are some laboratory studies (Carvalho & Lussi 2015; Carvalho & Lussi 2014; Lussi et al. 2008; Austin et al. 2011) that have shown the hardness of the enamel does not fully recover to pre-acid exposure. But this is unlikely to have a significant clinical impact otherwise erosion would be widespread and continually progressing in all individuals.

Laboratory studies have suggested that bulk tissue loss occurs when the organic matrix and crystal structure are removed (Eisenburger et al. 2004; West et al. 2000; Karlinsey et al. 2009). Bulk tissue loss is a feature of erosion rather than caries and the amount of loss tends to be in the region of several micrometres but as the condition deteriorates it might accumulate to millimetres. Once structural tissue loss occurs the enamel structure is removed and the potential for remineralisation is lost and the process becomes irreversible. The concern clinically is that if the cause of the acid is not prevented or the tissue loss proceeds at a high rate then dentine can be eventually exposed causing sensitivity and ultimately tooth loss.

Demineralisation is the process by which mineral in the tooth is removed but surface loss has not yet occurred, also known as surface softening. This demineralised layer probably extends a

few micrometres from the enamel surface (Addy & Shellis 2006). As the acidic solution diffuses into the enamel through the narrow pores it begins to demineralise the subsurface. Calcium ions and various phosphate anions are released into the solution and the hydrogen ions are consumed. Demineralisation stops within a small distance from the surface as the solution becomes saturated with the tooth mineral.

However, if other mechanical forces act on the partially eroded surface the lesion may progress. The combination of erosion and abrasion is believed to be one of the conditions when the early reversible lesion changes to one where tissue loss occurs. Laboratory studies modelling erosion followed by abrasion have reported significantly higher enamel loss compared to erosion-only (Eisenburger et al. 2003; Attin et al. 2007). But the assumption that erosion followed by abrasion always produces greater tissue loss is not straightforward. Vieira et al. in another laboratory study, reported that erosion followed by toothbrush abrasion produced a higher step height loss (approximately 6μm) but the difference was not significantly greater than erosion-only (approximately 4μm) (Vieira et al. 2006). The differences may be caused by differences in experimental protocols (e.g. the concentration and type of acid, remineralisation protocol, force of and amount of abrasion etc.). The overall concept that erosion and abrasion causes more wear is supported. However, the process is more complicated than initially thought. Figure 1 shows a transverse microradiography image of an early enamel erosive lesion, showing the softened sub surface layer. The clinical progression is unknown and whether the laboratory models that suggest the softened layer progresses to tissue loss or how, in favourable conditions, has the potential to be remineralised, is unknown.

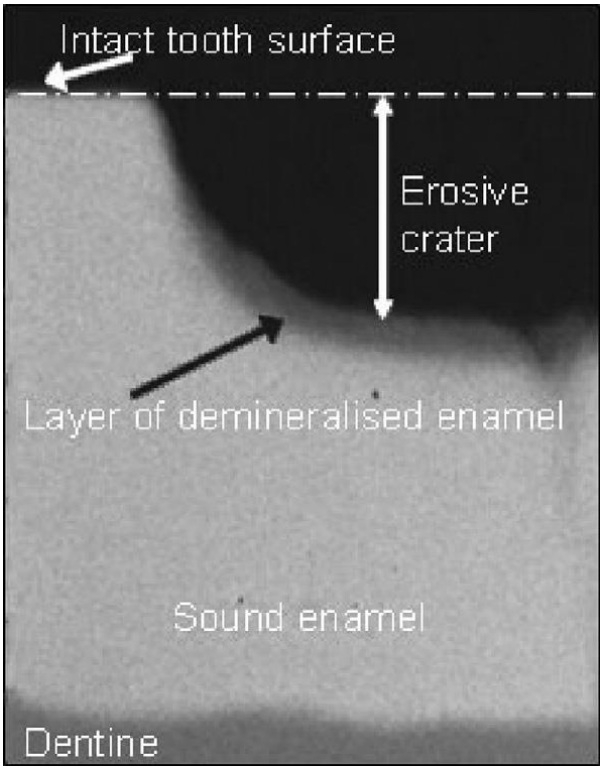

Figure 1 Transverse microradiography image of an enamel erosion lesion showing intact enamel, an erosive crater and subsurface demineralisation from (Elton et al. 2009)

Further studies investigating the inter-relationship between erosion and abrasion have been reported from other laboratory work including *in situ* studies (Eisenburger et al. 2003; Ganss et al. 2007; Attin et al. 2007; Wiegand, Kowing, et al. 2007). If it is accepted that eroded enamel is more susceptible to abrasive forces, there is a question of how long the enamel remains in a softened state? Laboratory studies suggest that enamel can remain softened for extended periods and therefore be more susceptible to mechanical wear for up to an hour (Jaeggi & Lussi 1999) or four hours (Lussi et al. 2014) after an erosive challenge. Both studies used the Knoop microhardness instrument to assess the effect of erosion by measuring the change in the depth of the indent after an abrasive challenge. Jaeggi and Lussi (1999) used an *in situ* study whereas the study by Lussi et al (2014) was a laboratory study. Zero and Lussi suggested, in a review paper, that tooth brush abrasion should be avoided immediately after an erosive challenge (Zero & Lussi 2005) but there remains no clear view on how long the surface of

enamel remains susceptible to abrasion in the clinical environment. Further work is needed to clarify this area.

1.7 Enamel

Enamel is the most highly calcified and hardest tissue in the human body, produced by cells of ectodermal origin. It covers the whole of the crown with varying thickness (Hall RC et al. 2000) and is composed mainly of the components found in Table 1 (Ten Cate et al. 2008):

- Pure Hydroxyapatite crystal, $Ca_{10}(PO_4)_6(OH)_2$, (approximately 95 % by weight)
- Water and organic matrix (approximately 5% by weight)
- Impurities-carbonate, fluoride and magnesium
- Trace elements-potassium, zinc, lead and copper (Curzon & Cutress 1983)

	% dry weight
Calcium	34-39
Phosphorus	16-18
Carbonate	2.0-3.6
Sodium	0.3-0.9
Magnesium	0.3-0.6
Chloride	0.2-0.3
Mineral	99
Organic	1

Table 1 Composition of enamel (% dry weight)

Enamel is made of super-assemblies of enamel prisms combined with varying amounts of interprismatic material (Hall et al. 2000). Within enamel there are gradients in the different constituents. For example, the mineral content of calcium and phosphorus increases, from the enamel/dentine junction to the surface, whilst carbonate and magnesium concentration decreases. Figure 2 shows the formula for the average composition of enamel mineral. It is believed that 10% of CO_3^{2-} replaces OH^- ions rather than PO_4^{3-} and that fluoride is not included

due to its low concentration in bulk enamel compared to surface enamel (Featherstone & Lussi 2006; Driessens et al. 2010). These substituted ions increase strain within the crystals, which increases its solubility.

$$[Ca_{8.9}Na_{0.3}Mg_{0.14}K_{0.01}\square_{0.65}]\ [(PO_4)_{5.1}(HPO_4)_{0.4}(CO_3)_{0.5}]\ [(OH)_{1.08}(CO_3)_{0.05}Cl_{0.1}\square_{0.77}]$$

Figure 2 Formula for the average composition for enamel mineral, where □ indicates vacancies in the crystal lattice

1.7.1 Hydroxyapatite crystal

The enamel prism is the basic structural unit, made of several million hydroxyapatite crystals packed into long, thin rods; these prisms have various shapes, orientations and patterns, depending on the maturity of the enamel and location within the tooth. The hydroxyapatite crystal is the principle mineral component of enamel. It has a hexagonal cross section with a width of approximately 70nm and a thickness of approximately 25 nm. The length is variable and can stretch up to 1000nm, Figure 3.

The orientation of the prisms in mature enamel is determined by the direction of movement of secretory ameloblasts in early enamel formation. Hunter-Schreger bands (groups of 10-13 prims) are formed in the inner one half to two thirds of enamel by curvature of the prisms. The complicated prism arrangement within the Hunter-Schreger bands is thought to reduce the propagation of fractures. Due to the variation in prism orientation, inner enamel is relatively porous.

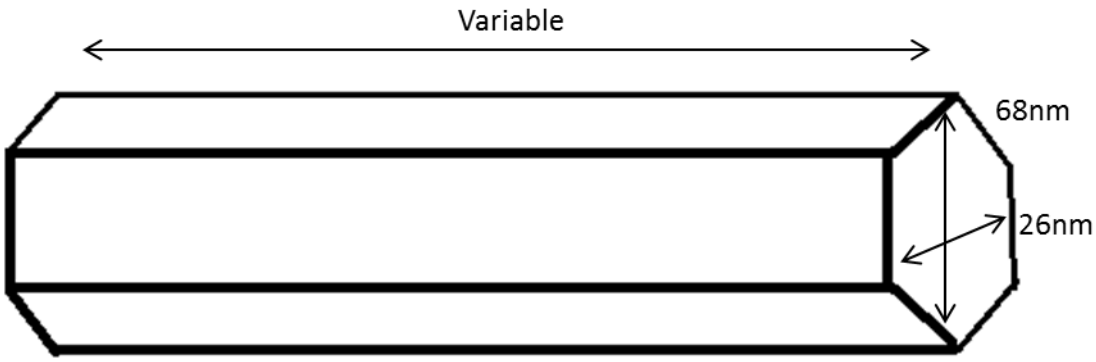

Figure 3 Impure hydroxyapatite crystal approximate dimensions

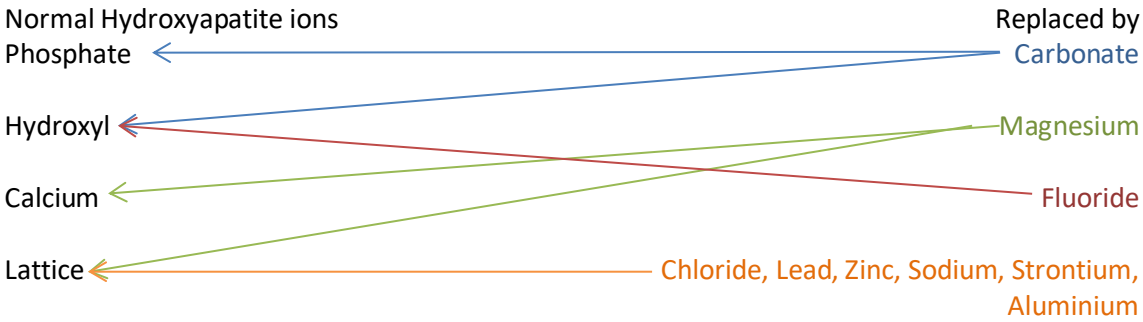

Figure 4 The ionic species that replace the 'normal' ions in hydroxyapatite

Three main patterns are present in the cross-sectional appearance of enamel (Boyde A 1965; Boyde A 1978; Boyde A 1989). Type I has prisms with complete boundaries, well defined inter-prismatic regions and appear circular. Type II has prisms aligned in parallel rows and appears to have incomplete outlines. Type III, the most common in human enamel, has prisms arranged in staggered rows, it appears as a 'keyhole' or 'horseshoe'. Aprismatic enamel is present at the surface of the tooth and has a thickness of up to 100µm, depending upon its location on the tooth. The crystals are parallel to each other, perpendicular to the surface and lacking prism boundaries. These properties make it more highly mineralised than subsurface enamel. It is thought to arise by the loss of the Tomes process in enamel formation.

Sites of abrupt change in crystal orientation cause the formation of the pores with increased porosity at the junctions. The pores affect the mechanical and optical properties of enamel.

The pores in outer enamel tend to be separate whereas those in the inner enamel have interconnecting zones, which form a three-dimensional network. The presence of pores may allow the movement of acid into the bulk of the tissue and ions to flow into and out of the enamel, which can cause de/re-mineralisation of the subsurface.

The orientation, pattern and microporosity of enamel is determined by the prism size and all these factors affect the properties of enamel (Mabilleau et al. 2010). Eimar et al. investigated the effect of prism size on hardness and found that teeth containing a shorter prism size were harder compared to those with longer sized crystals and therefore were more resistant to tooth wear and erosion (Eimar et al. 2012).

The porous composition of enamel allows for the natural movement of minerals into and out of the enamel by diffusion. Ionic species can replace the 'normal' ions found in hydroxyapatite crystals (Figure 4) and so the shape can be distorted. The core of the crystal has a higher proportion of magnesium/carbonate ions and therefore, is more soluble (Nelson 1981) than the edges.

Water is present in enamel by approximately 2% by weight and lies between the crystals and organic material. It also forms a hydration layer on the crystal surfaces. Its distribution through the enamel structure is important, as it is present in sufficient quantity for ions and trace elements to travel through it. Also, there is organic matrix present in the enamel that consists of amino acids, peptides and carbohydrates.

The structural features of enamel contribute to its susceptibility to erosion. The porous structure and presence of water allows the movement of acids into the enamel, which increases the potential for erosion. Aprismatic enamel at the surface has fewer pores and this decreases the susceptibility. Once the aprismatic enamel has been removed, the underlying surface is more susceptible to erosion (Carvalho & Lussi 2015). The substitution of minerals

into and out of hydroxyapatite can be beneficial (remineralisation) or detrimental (demineralisation) in the protection against erosion. Deciduous and permanent teeth show different susceptibility to erosion and some authors have put this down to the differences in their structure (Lussi et al. 2000; Wang et al. 2006).

1.8 Human v Bovine

Ideally, enamel obtained from humans should be used as the experiments would be closer to the clinical conditions. This however is often difficult to achieve, as sourcing sufficient human enamel can be challenging. To overcome the supply issues different sources of enamel have been used including primate, bovine, swine, equine and shark. Of these, bovine is most commonly substituted (Yassen et al. 2011). Bovine teeth are larger in size, easier to collect and so are considered, by some, to be suitable for laboratory or clinical studies. But both human sourced and bovine enamel have disadvantages. There are differences in opinion about the appropriateness of using bovine or human teeth for clinical studies. For *in situ* studies, some people would not allow bovine teeth to be used in their mouth whilst others would not accept human teeth. A major concern is the effective sterilisation of enamel. There are several methods such as gamma irradiation, steam autoclaving, sodium hypochlorite and povidone-iodine. Amaechi et al. investigated these methods and concluded that they were all effective to sterilise enamel and none of them caused significant demineralisation compared to a control(Amaechi et al. 1998). The recommended method from Kings Health Care Partners are to use sodium hypochlorite immersion for at least 2 hours, other bodies suggest the use of gamma irradiation as this might have the least effect on demineralisation but would also ensure the denaturing of prions. There are also differences between their chemical and physical properties which may influence the choice of using them in a study.

1.8.1 Chemical and Physical Composition

Yassen et al. reviewed the literature comparing human and bovine teeth from 68 papers between 1953 to 2010 (Yassen et al. 2011) and concluded that chemically, human and bovine enamel were similar. Both have a similar calcium/phosphate ratio, calcium and carbonate content but differ in the distribution of calcium, with more uniform distribution and higher protein content observed in bovine enamel. Physically, bovine teeth are larger and flatter compared to human teeth, which makes preparation of bovine teeth easier for laboratory studies.

Putt et al. reported no difference in polishing properties (Putt et al. 1980), the remineralisation/ demineralisation reaction (Feagin et al. 1969) and fluoride uptake (Gwinnett et al. 1972) between the two tissues. However, Field et al. reported that under the same polishing procedures the microhardness and roughness of bovine enamel was smoother and harder than human enamel (Field et al. 2014). But contradictory results were reported comparing the Knoop microhardness data between human and bovine teeth. Souza-Gabriel et al. showed that human teeth had significantly lower Knoop microhardness values than bovine teeth (Souza-Gabriel et al. 2010) whereas Turssi et al. showed that human teeth had higher values, although this was not significant (Turssi et al. 2010). Under erosion-only conditions, Attin et al. reported bovine enamel to erode more than human enamel (5µm v 2µm) however under abrasion-only, there were no differences (Attin et al. 2007). The challenge with these results is that the model conditions varied between the studies and so it is impossible to directly compare them. On balance, there is little to distinguish between the two and so the decision to use one or the other may be based on convenience of supply or personal preference.

Table 3 contrasts human to bovine teeth. Bovine teeth are larger with more consistency in the surface and physical properties (Gonçalves et al. 2012; Wang et al. 2012) and can be obtained in large numbers (Shellis et al. 2011; Young & Tenuta 2011).

	Human	Bovine
Advantages	Clinically relevant	Easy to obtain in large quantities in a good condition More uniform composition Relatively large flat surface No defects No ethical approval necessary
Disadvantages	Difficult to obtain in large quantities in good condition (no cracks, caries free, no composite) Difficult to control source and age Relatively small and curved surface area Varying thickness Time consuming ethical approval	Not clinically relevant

Table 2 Advantages and disadvantages of using human and bovine enamel for *in vitro* studies

The ability to access bovine enamel allows for greater numbers, larger sizes and makes them more convenient to use in *in vitro* and *in situ* studies. However, human enamel may be the preferred choice for studies as this may offer a better representation of the clinical environment. Until human teeth or enamel can be grown *in vitro*, which recent research has started to show promising results (Angelova Volponi et al. 2013), using extracted human teeth is the best alternative for accurate conclusions. Our research group has elected to use human teeth due to the relative ease of collecting and obtaining samples within the hospital setting.

1.9 Saliva

Saliva is a biological fluid secreted by three major salivary glands; the parotid, submandibular and sublingual glands into the oral cavity. It is multifunctional with antibacterial/viral/fungal properties as well as aiding in digestion and buffering. It has been thought that it could be a highly important factor in erosion as it interacts with the acids by; diluting, clearing or

neutralising actions (Buzalaf et al. 2012). It also provides a protective layer in the form of a pellicle and has a role in remineralisation.

Formation of a salivary pellicle occurs within the first few moments after brushing and consists of a protein rich barrier. This thin layer, between 0.3 to 1.06µm in thickness (Amaechi et al. 1999; Hannig et al. 2001), acts as a barrier and prevents acids from demineralising the tooth and also prevents the dissolution of mineral ions and anions (Ca^{2+}, PO_4^{3-}, OH^-) from passing into the saliva (Attin 2006). This layer is supersaturated with calcium and phosphate with respect to hydroxyapatite and so allows favourable conditions for remineralisation by diffusion of the ions back into the enamel. The super-saturation is maintained by the saliva (Smales et al. 2009; Buzalaf et al. 2012).

1.9.1 Natural

Natural saliva mainly consists of water. It also contains a variety of organic and inorganic compounds such as enzymes, proteins, glycoproteins, matrix metalloproteinases, bicarbonate, calcium and phosphate. The major proteins that have been investigated for their effect on erosion are statherins and mucins. In the oral cavity, in the presence of saliva the tooth surface is covered by an 'acquired pellicle' which is an organic film free of bacteria containing over 130 different proteins (Buzalaf et al. 2012).

1.9.2 Artificial

Artificial or synthetic saliva is used as a substitute to natural saliva. As natural saliva can be difficult to obtain and store in sufficient quantities, artificial saliva helps to overcome this problem. Clinically, it is used for patients if they suffer from a dry mouth resulting from low salivary flow. It attempts to recreate the mineral content of natural saliva but cannot match the complexity and individual variation found in natural saliva. Proteins, minerals and buffers among many other components can be individually added to water to mimic natural saliva. For

instance, to increase thickness, compounds such as sodium carboxymethyl cellulose can be added but cannot at the present time substitute natural saliva completely.

For research purposes, the main choice with artificial saliva is whether to include proteins. Artificial saliva normally include a variety of salts, bases, minerals and buffer such as KH_2PO_3, Na_2HPO_4, KCl, $MgCl_2$, HEPES and $CaCl_2.2H_2O$. Studies have shown that artificial saliva both with and without proteins has the ability to partially remineralise initial erosive lesions in enamel (Ionta et al. 2014). But the benefit of using artificial saliva is that it can be made to a consistent formulation, in large quantities, and so allows greater standardisation in studies.

There is no universally agreed standard formula for artificial saliva used in *in vitro* erosion studies. This means that the differences in formulation makes accurate comparisons between studies difficult, as it is unknown what effect any interactions between the constituents have on the outcome. But it depends on why the artificial saliva is used. If pellicle formation is necessary, the presence of proteins is a necessary component whereas if the hypothesis focuses on remineralisation they may not be required. Artificial saliva can be made in the laboratory (Ionta et al. 2014) or bought commercially (Aykut-Yetkiner et al. 2014) and are variable. This wide array of components and mixtures was investigated by Ionta et al., when they compared five artificial saliva solutions on remineralisation (Ionta et al. 2014). Table 3 shows a summary of the different formulas investigated, displaying the compounds and the amounts used. The study concluded that all the solutions produced significantly greater ($p<0.001$) remineralisation compared to a control, with formulation "3" producing the highest amount of remineralisation determined by microhardness change (Amaechi & Higham 2001; Eisenburger et al. 2001). Eisenburger et al. used the same saliva formulation "3" and reported a complete re-hardening of the enamel surface after 6 hours of immersion (Eisenburger et al. 2001). Aykut-Yetkiner et al. investigated 15 commercially available artificial saliva solutions and their effect on *in vitro* erosion and reported that four of the sprays increased erosion.

However, this was not surprising as the solutions had a low pH and contained citric acid. It highlighted that if artificial salvia solution was used, the contents must be fully listed as, in the case of the 4 sprays. However, all these studies are laboratory based and involve long remineralisation times but what remains unknown is the impact this has clinically. For the present time the laboratory studies hopefully predict the clinical situation but perhaps the process is lengthened.

It is common to find artificial saliva being reported in laboratory studies (Kielbassa et al. 2001; Amaechi & Higham 2001; M. Eisenburger et al. 2001; Rodriguez & Bartlett 2010). The difference between artificial saliva and natural saliva when used in laboratory studies will depend upon the hypothesis being investigated. Ideally, all *in vitro* studies should use natural or artificial saliva as this replicates the clinical situation and might make the findings more useful. For those studies that use saliva as a lubricant the impact of the difference between artificial and natural is not likely to be significant. However, when used for remineralisation the importance of proteins becomes greater and natural saliva should be considered. The balance between selecting natural or artificial saliva depends upon the hypothesis.

	Formula 1 /g	Formula 2 /g	Formula 3 /g	Formula 4 /g	Formula 5 /g
KH_2PO_4	0.33	0.33	0.544	0.738	0.326
$NaHPO_4$	0.34	0.34			
KCl	1.27	1.27			
NaSGN	0.16	0.16			
NaCl	0.58	0.58		0.381	
$CaCl_2$	0.17	0.17			
NH_4Cl	0.16	0.16			
Urea	0.2	0.2			
Glucose	0.03	0.03			
Ascorbic Acid	0.002	0.002			
Mucin	2.7			2.2	
$CaCl_2.2H_20$			0.1029	0.213	0.166
MgCl			0.04066		
HEPES (acidic form)			4.766		
KCl			2.2365	1.114	0.625
Methyl-p-hydroxybenzoate					2
Sodium carboxymethyl cellulose					10
$MgCl_2.6H_20$					0.059
K_2HPO_4					0.804
pH	7	7	7	7	7

Table 3 Table showing the constituents of the different formulations of artificial saliva and the amounts used (g in 1000 ml of distilled water) in the study by (Ionta et al. 2013)

1.10 Chemistry of Acids

The most common way to define an acid is with the Bronsted-Lowry definition of 'a substance or molecule that can act as a proton donor'. Conversely a base by this definition is 'a substance or molecule that can act as a proton acceptor'. Acids can also be described as 'Lewis acids'; a Lewis acid is a substance or molecule that can accept a pair of electrons whereas a Lewis base is a substance or molecule than can donate a pair of electrons. A final way to describe an acid is as an 'Arrhenius acid', which is defined, as a substance or molecule that increases the concentration of hydronium ions (formed by dissociation of water into hydronium, H_3O^+ and hydroxide, OH^- ions). While all are correct and have their advantages in explaining certain reactions, for dental research the most applicable definition would be the Bronsted-Lowry as it

uses protons to describe acid behaviour, which is, more applicable to dietary acids seen in food and drinks.

Acids can vary in strength and their strength is dependent on a variety of factors, which are explained in more detail in the sections below. Acids react with bases and particular metals to form salts. There are several groups of acids ranging from organic, inorganic, mono and polyprotic.

1.10.1 Organic

Organic acids contain carbon within the molecule. In solution they tend to be partially dissociated and classed amongst the weak acids and their acid strength is determined by the stability of its conjugate base. The stability of the base arises from its ability to stabilise the negative charge on the molecule after the loss of a hydrogen atom. This can be via mechanisms such as the inductive effect, resonance and level of conjugation within the molecule. Organic acids are naturally found in fruit and fruit products with citric and malic acid being the most common. They are also added to soft drinks and other food-stuffs. The quantities of these acids rely on several factors such as ripeness, seasonal variability or manufacturer preference in response to taste or preservation calculations.

The most common organic acids are carboxylic acids, citric, malic and lactic acids. Their acidity arises from their carboxyl group, which is a carbon double bonded to an oxygen atom and then single bonded to a hydroxyl group and is often written as '–COOH'. Citric acid would be denoted as 'CH_2(COOH)COH(COOH)CH_2(COOH)', and from this there are three carboxyl groups that can each donate a hydrogen atom contributing to the acidity of the molecule. However, the ability of the molecule to lose the protons depends on several properties of the molecule and the environment; such as resonance, delocalisation, pH and temperature.

1.10.2 Inorganic

Inorganic or mineral acids are acids whose molecule does not contain carbon. In solution they can be either fully or partially dissociated which gives them a larger range in acidic strength, from very weak (e.g. boric acid) to very strong (e.g. sulphuric acid). Hydrochloric and phosphoric acid are the most relevant inorganic acids in relation to erosion. Hydrochloric acid is found in the stomach and is one of the main acids causing erosion when gastric contents enter the oral cavity (Scheutzel 1996; Büyükyilmaz et al. 1997). It has a low pH (1-2) and is fully dissociated into hydrogen ions and chloride anions in solution. Phosphoric acid is a weaker acid found mainly in soft drinks and is widely used in the food industry as it is a cheap acidulant. They are also used as in the manufacture of fertilisers, plastics and other substances.

1.10.3 Monoprotic

Monoprotic acids can only donate one proton per molecule. Hydrochloric acid is the main monoprotic acid responsible for the intrinsic source of erosion and which is completely dissociated in solution. Another monoprotic acid is nitric acid**,** which is predominantly used in the production of fertilisers and explosives and is not relevant to dental erosion.

1.10.4 Polyprotic

Polyprotic acids are acids that can donate more than one proton per molecule. Acids, such as citric and phosphoric acid can donate up to three protons. In solution they exist as a combination of un-dissociated and partially dissociated acid molecules and hydrogen ions in equilibrium, whose relative concentrations are determined by the addition of an acid, buffer or solvent. This disrupts the balance, shifting the equilibrium and concentrations of the molecules and therefore the erosive potential. After the dissociation of the first proton, subsequent loss of further protons becomes more difficult as the new acid is weaker than the original and stability of the conjugate base is reduced. Also affecting the concentrations of the different

species are properties of the molecule (which are described below) and rates at which they are consumed.

1.10.5 Dissociation and equilibrium

In solution, acids tend to exist in equilibrium. Figure 5a shows the general equation for the dissociation of an acid in solution. The acid 'HA' dissociates into a proton 'H^+' and its conjugate

a) $HA \leftrightharpoons H^+ + A^-$ b) $K_a = \frac{[A-][H+]}{[HA]}$

Figure 5 a)General equation for dissociation of an acid in solution where HA = acid and A^- = conjugate base b) calculation of acid dissociation constant (K_a)

base (A^-). A stronger acid will have a greater dissociation and this is due to the stability of the conjugate base. When a proton is lost, for example in the reaction in Figure 5, the molecule that lost the proton has an extra pair of electrons (a negative charge) so it becomes highly reactive and attempts to recover the proton. However, if this charge is stabilised then it is less reactive. Stabilisation of the negative charge can be achieved via the inductive effect, resonance or delocalisation. A strong acid, like hydrochloric acid, is effectively fully dissociated and so the equilibrium is pushed fully over to the right (of the equation) with little un-dissociated acid left in the solution. For weaker acids, such as citric and phosphoric, there is also an equilibrium but it lays slightly to the right. The amount of dissociation is measured by the Ka, the acid dissociation constant (Figure 5b).

The acid dissociation constant is temperature dependant; if the reaction is endothermic (such as dissolving citric acid in water) then the acid dissociation constant will increase with temperature meaning that at higher temperatures the solution is more acidic.

The inductive effect is achieved by the non-uniform distribution of electrons towards the more electronegative of two atoms. The more electronegative atom will draw electron density from

the atom with the lone pair of electrons through the covalent bond, thus slightly reducing the effect of the negative charge and making the molecule slightly less reactive. Resonance or delocalisation of electrons makes the molecule less reactive by spreading the charge when suitable molecules/functional groups are present that can accept a pair of electrons. The lone pair of electrons that are present after the loss of a proton are effectively moved to another atom within two bonds from the original. In reality however the 'true' structure lays between these two extremes with an area of delocalised electrons between the 3 atoms and 2 bonds.

1.10.6 pH and pK_a

The pH provides information on the availability of hydrogen ions in a solution. A pH of 7 is seen as neutral, below this value it is 'acidic' and above this value it is seen as 'basic'. Enamel dissolution increases with a decreasing pH for all acids (Barbour et al. 2003).

The Ka/pKa (acid dissociation or equilibrium constant) represents the ability of an acid to maintain its acidity, referred to in dental literature as the 'buffering capacity'. Figure 5b shows the equation to calculate the acid dissociation constant of an acid. A larger Ka value indicates greater dissociation and therefore a stronger acid. Ka is often expressed as pKa, which is the negative log of Ka and makes comparison easier due to the large orders of magnitude of Ka. With pKa a lower value indicates greater dissociation and therefore greater acid strength.

Monoprotic acids have only one pKa value as they can only donate one proton. Polyprotic acids have several pKa values. If a molecule can lose 3 protons then it would have 3 values often denoted as pKa1 for the loss of the first proton then pKa2 and pKa3 for the remaining protons. The pKa values increase for the loss of further protons on the same molecule as subsequent losses of protons from the molecule make the new molecule a slightly weaker acid.

1.11 Properties of acids

The pH, titratable acidity, buffering capacity (K_a/pK_a), calcium chelating properties and calcium/phosphate concentration are factors affecting the potential of a dietary food or drink to cause erosion (Zero 1996). Acids can also be characterised by their taste, which is sour, compared to bases, which tend to be bitter. Acids also turn blue litmus paper red. These tests however don't provide any useful additional information with regards to strength or composition of an acid. As acids are added to food and drinks for preservatives and also to alter taste, the taste test maybe useful, however quantifying this would be difficult.

1.11.1 Calcium/Phosphate concentration

The calcium and phosphate content of solutions affect the concentration gradient of minerals at the tooth surface layer which in turn affects the erosive potential (Lussi & Jaeggi 2006). When a solution is under-saturated, with respect to tooth minerals, calcium and phosphate can diffuse out of the enamel, causing erosion. Generally, at a low pH, a higher concentration of calcium and phosphate is needed to saturate the solution with respect to hydroxyapatite, increasing the erosive potential. However yoghurt, which has a low pH, causes little erosion as it is supersaturated with respect to hydroxyapatite.

Calcium is a group two, alkaline earth metal. Generally the salts are colourless in solution and are fairly soluble in water. In solution it exists as the Ca^{2+} ion. The citrate ion (formed after loss of hydrogen from citric acid) can chelate with the calcium ion and the calcium's affinity to the citrate ion becomes stronger as more protons are lost. Phosphate is an inorganic salt of phosphoric acid and is present in solution after dissolution of enamel. In aqueous solution it can exist in four forms (phosphoric acid, dihydrogen phosphate, hydrogen phosphate and phosphate) depending on the pH of the solution. In the case of dental research it is mainly in the phosphate form. It too can chelate to calcium ions.

1.11.2 pH and Critical pH

The pH is the measure of the concentration of hydrogen ions available in the solution. Often it is used as the sole indicator for a food or drinks ability to cause erosion. The critical pH is a pH value that is often used in dental literature to say when erosion in a solution starts, but it is not a term that is commonly used in chemistry. The critical pH is defined as the 'pH value at which a solution is just saturated with respect to a specific solid' (Lussi & Carvalho 2014). It occurs when the solution is just saturated with minerals found in enamel and, in caries research, is often given a value of approximately in the range of 5.5-5.7 (Touyz 1994). Above the pH value, erosion does not occur as the solution is supersaturated with enamel minerals, and below, erosion can occur as the solution is under-saturated (Dawes 2003). The value of 5.5 finds the origin from the caries model and relates to the calcium and phosphate concentrations found in plaque fluid; it seems to have been incorrectly adopted for erosion. In erosion, the 'critical pH' also varies across a range but will depend on the solubility of the enamel, constituents of the solution such as calcium and phosphate concentration, chelating properties of the acid and the buffering capacity. Dawes suggested that the critical pH is inversely proportional to the calcium and phosphate concentration (Dawes 2003; Lussi & Jaeggi 2006). For example, low pH products such as yoghurts (naturally containing high concentrations of calcium and phosphate) or orange juice supplemented with calcium and phosphate have been shown to cause minimal erosion and surface softening (Lussi & Jaeggi 2006; Larsen & Nyvad 1999). Conversely higher pH solutions could still cause erosion through chelation. This was shown partly by Hsu et al. who used high citrate concentrations, around pH 4-6, and showed qualitatively more erosion (Hsu et al. 1994).

In summary, the term critical pH is a helpful concept as it allows the researcher a quick and convenient way to determine on a basic level if a solution has a possibility to cause erosion. Any extremes of pH above or below the 'critical pH' will probably follow the expected trend but as the pH becomes closer to pH 5.5 then the interaction with other variables becomes

more prominent and the conclusions need to be carefully interpreted and further analysis of the solution (calcium/phosphate concentration) must be performed. Overall, it provides a crude analysis of a solution's potential to cause erosion and provides a starting point for further analysis.

1.11.3 Titratable Acidity

The term titratable acidity was historically used in the dairy and wine making industries. It was used to measure the total amount of lactic acid (for dairy) or tartaric acid (for wine) and would be expressed as a % of the desired acid. Both provide a measure of the total acidity of a solution. However in dental research, the concept is used as a measure of the hydrogen ion concentration or pH and is used to indicate the strength the solution.

The titratable acidity is calculated by the amount of base required to raise the pH of an acidic solution. A higher titratable acidity increases the potential for erosion. The measurement of the titratable acidity varies between authors. For example, Cochrane et al. reported using 0.05M NaOH solution to increase the pH of 20mL of an acidic solution to pH 7 (Cochrane et al. 2012). On the other hand, Lussi et al. reported using 0.5M NaOH solution to increase 10mL of acidic solution to pH 7 (Lussi et al. 2012). Both assessments reported the titratable acidity in 'mmol OH^-/L' but produced different results. Coca-Cola reported by Cochrane et al. and then Lussi et al., to have different values of 23.36 and 17.5mmol OH^-/L respectively. Because of this the values given for titratable acidity must be carefully interpreted particularly when different techniques are used as some authors do not provide all the relevant details such as concentration of sodium hydroxide and final pH, making comparison impossible.

If the base used to titrate the acid is too weak, then the risk of diluting the initial solution increases. This makes the calculation incorrect, as the concentration and constituents of the solution you are titrating would have been diluted by the addition of a large amount of base.

1.11.4 Buffering Capacity

Buffering capacity of an erosive solution is the ability to resist change in pH and is related to the acid dissociation constant. It is seen as the driving force for demineralisation and the greater the buffering capacity of a solution, the longer it will take for it to be neutralised by saliva and therefore it will cause more erosion (Lussi et al. 2012). Buffering capacity can be defined in different ways; the amount of acid/base required to give a significant change in pH or the amount of acid/base required to change the pH of one litre of solution by one pH unit. Calculating this becomes further complicated as erosive solutions contain several species, whose buffering capabilities need to be accounted for as they can influence the outcome.

$$\beta = \frac{\Delta C}{\Delta pH}$$

Figure 6 Equation for calculating the buffering capacity (β), where ΔC is volume of base used and ΔpH is the change in pH

Figure 6 shows a simple equation for calculating the buffering capacity. It requires data from a titration (typically a titratable acidity measurement), which itself is subject to much variation and inconsistent reporting.

Clinically, the buffering capacity of a solution will be altered once it enters the oral cavity. The dilution with saliva and change in temperature affects this property. Also, pure acids are rarely consumed and are consequently part of a solution that contains other ingredients all of which have the capacity to alter the value. For this reason, *in vitro* studies using pure acids do not accurately represent the clinical environment, meaning that conclusions must be carefully considered.

The constituents of saliva and therefore their buffering capacity vary between patients. In healthy individuals saliva has a high buffering capacity as it contains proteins, some of which prevent calcium deposition and so make the saliva calcium rich. It has been shown that

patients whose saliva has a lower buffering capacity maybe more susceptible to erosion (Piangprach et al. 2009; Aiuchi et al. 2008; Amaechi et al. 1999).

Buffering capacity is widely accepted as a measure of a solution's ability to cause erosion, as it measures the ability to resist a change in pH. However, it should not be used in isolation and should be used with other measures such as pH, titratable acidity, calcium and phosphate concentration to give a more complete picture and better understanding of the solution.

1.11.5 Chelation

The chelating property is the ability of anions in a solution to bind or complex to the calcium in enamel removing it from the surface and passing it into the solution resulting in erosion, or by chelation of the Ca^{2+} in solution, maintaining the concentration gradient. The pH of the solution and the structure of the anion will determine how much chelation occurs. Citric acid contains the citrate anion and is able to chelate to the calcium ion strongly compared to other acids due to its favourable size and pKa values (Featherstone & Lussi 2006), which increases the potential to cause erosion.

The chelation potential of the solution depends on the constituents. Citric, and to a lesser extent phosphoric acid, can chelate to the calcium ions present in enamel. The extent of the chelation depends on the environment of the acid molecule. For citric acid, at low pH, chelation to calcium ions is low as its ability to bind to and hold onto the calcium is reduced. This is due to hydrogen ions still attached to the citric acid making its shape unfavourable for chelation. As the acid molecule loses its hydrogen ions it becomes more energetically favourable for the calcium to chelate with it as it has more electrons available for bonding, Figure 7, forming a three dimensional electrostatic interaction.

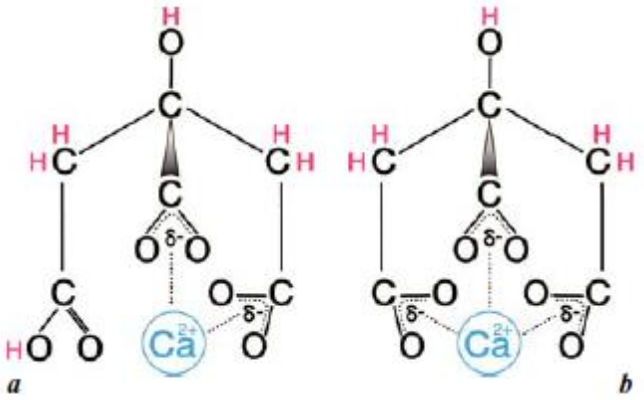

Figure 7 Figure showing chelation of calcium by citrate ion after it has lost a) 2 hydrogen and b) 3 hydrogens from (Shellis et al. 2014)

For citric acid, the 3 pKa values (25°C) at which it loses its first, second and third hydrogen ions are 3.13, 4.76 and 6.40. This informs us that at low pH's (<2) only hydrogen ion donation is causing erosion however at higher pH's (>7), when all the hydrogen ions have been lost, then only chelation occurs. At an intermediate pH, both mechanisms occur.

1.12 Erosive potential of food and drinks

Whilst the effects of individual acids on erosion can and have been studied, the reality is that these will rarely, if ever, be consumed in isolation and will in fact be part of a food or drink. *In vitro* investigation of food and drinks on erosion are therefore often performed. Lussi et al. investigated the erosive effect of 60 commercial products on enamel for microhardness, and assessed the pH, concentration of calcium, phosphate and fluoride, titratable acidity to pH 7, buffering capacity, the degree of saturation with respect to hydroxyapatite and fluorapatite (Lussi et al. 2012). They reported that a significant effect on tooth erosion was found for pH, buffering capacity and calcium and fluoride concentrations. Of the solutions investigated the pH ranged from 2.39 – 7.51 with coffees and teas having the highest pH values and energy and soft drinks having the lowest. The buffering capacities ranged from 2 – 200mmol/l x pH, with high values for yoghurts, fruits and salad dressing and low values for water, tea and coffee. Calcium and fluoride ranged from 0.12-56.33mmol/L and 0.01 – 1.63mg/L (Lussi et al. 2012).

The extra information allows for better understanding and interpretation of the results. For example, Berocca fizzy vitamin c tablets produced less surface softening than Alka-Seltzer fizzy vitamins c tablets. Restricting the analyses to the pH, Berocca (pH 4.24), should produce more enamel softening than Alka-Seltzer (pH 5.51) due to its lower pH. However, Berocca tablets had much higher concentrations of calcium and phosphate (15.2 & 0.03mmol/L) compared to Alka-Seltzer (2.04 & <0.01mmol/L), which explains the reduced erosive potential of the Berocca tablets.

Whilst it would be nice to perform this level of analysis for every solution that is used, practically, it would be difficult. It is also dependant on the equipment and expertise available. This study shows that conclusions made about the potential of an erosive solution based on one variable, such as pH or the buffering capacity (as is often reported), is wrong and in fact all of the constituents of an acid or solution need to be considered. The calcium/phosphate concentration, pH, titratable acidity, buffering capacity and chelation allow some prediction and explanation for dietary substances to cause erosion. However a prediction cannot be based on one factor as there are complex interactions between them. The influence of each factor on the fluid layer contacting the tooth surface should also be considered as this determines whether erosion will occur or not (Lussi & Jaeggi 2006).

Another study set out to quantify the amount of citric acid found in lemon juice, lime juice and commercially available fruit juice products. Whilst this study was not directed related to dentistry, its findings are interesting and relevant as citric acid is one of the main causes of erosion. Of all the products tested, Penniston et al. found that lemon juice contained the highest amount of citric acid (48g/L) and that Crystallized lemon, True Lemon commercial product contained the lowest amount (0.92g/L) (Penniston et al. 2008). This study also shows that there is large differences in the constituents (citric acid for this study) in commercial

products and further highlights the importance of reporting the details of an experiment in detail and correctly.

1.13 Fluoride

Fluoride is one of the main active products responsible for the decline in caries. More recently, its effect on the prevention of erosion has been investigated.

1.13.1 Chemistry

Fluorine is a highly reactive halide, group seven, non-metal. It is found naturally as fluoride at low levels in drinking water and foods. In dentistry, many types of fluoride are used such as sodium, stannous and amine fluoride, titanium tetrafluoride and sodium monofluorophosphate. Varying concentrations from 110ppm to 22600ppm have been investigated but the main emphasis has been on its effect on caries. However, more recently studies have investigated the effect of fluoride on the erosion process through addition into food/drink (Magalhães et al. 2014; Larsen 2001) and conventionally through fluoride-containing products (Hove et al. 2014; Faller & Eversole 2013; Carvalho & Lussi 2014).

1.13.2 Mode of action on dental surfaces

In terms of caries prevention, fluoride is thought to produce anticaries and cariostatic effects mainly by reducing the amount of demineralisation (by substitution within the tooth mineral), promoting remineralisation and by inhibiting the metabolism of oral bacteria which prevents acid production. Keeping a low, but elevated fluoride ion concentration, next to the tooth surface is believed to be important to achieve caries control (Duckworth 2013; Fejerskov et al. 1981).

In terms of erosion, the mode of action differs and there are two theories as to how fluoride protects against erosion. The first is the formation of a protective layer by precipitation of mineral salts. This could be a CaF_2 layer or more resistant polyvalent metal-fluoride layers with

metal ions such as tin (Ganss et al. 2004; Hercules & Craig 1978; Ganss et al. 2010) or titanium (Wei et al. 1976), on enamel. The second is substitution of the Ca^{2+} ion by metal ions e.g. Sn^{2+}, a few micrometres from the enamel surface (Schlueter et al. 2009). Both theories reduce the susceptibility of enamel to an erosive attack by temporarily protecting the underlying enamel. However, in highly acidic conditions this potential for protection maybe overwhelmed by the strength of the acid. Laboratory studies suggest the most important mode of action would be the formation of the metal-fluoride layer as it acts as a physical barrier to acid demineralisation. Theoretically, if the acid can be prevented from reaching the enamel surface it will prevent demineralisation and softening, so the enamel surface would not be in a vulnerable state to be affected by abrasive forces.

1.13.3 Sodium Fluoride (NaF)

Sodium fluoride is a monovalent fluoride compound and can supply one fluoride ion per sodium fluoride molecule. Sodium fluoride, investigated through *in vitro* studies, has shown to be effective against erosion compared to fluoride free controls. Ganss et al. in a laboratory study, reported that after erosive cycling, a 250ppm NaF solution produced significantly less erosion (13.2μm ±21.7) compared to a control (21.4μm ±19.4) (Ganss et al. 2008).

There are studies comparing experimental solutions containing sodium fluoride with the addition of sodium trimetaphosphate (Pancote et al. 2014; Manarelli et al. 2011), chitosan (Carvalho & Lussi 2014), monoalkyl phosphate (Jones et al. 2013) and xylitol (Rochel et al. 2011). All these studies reported that the addition of sodium fluoride reduced the amount of erosion to varying degrees compared to a placebo. Comparing the data between the studies is difficult due to the differences in experimental protocols. For example, in four of these studies, the fluoride concentrations tested varied from 100 to 9000ppm and in all 16 different sodium fluoride compounds were tested. All the studies measured the step height loss. However, due to the variations in the experimental protocols and fluoride products, it is not possible to

directly compare the data. However, the overall trend suggests that sodium fluoride provides some degree of protection.

1.13.4 Stannous Fluoride (SnF_2)

Stannous (tin) fluoride is a polyvalent fluoride compound, supplying two fluoride ions per stannous fluoride molecule. Stannous fluoride has been shown, *in vitro*, to offer between 55% (Ganss et al. 2011) and 95 % (Young et al. 2006) reduction of enamel loss when compared to a water control. An *in situ* study comparing the effect of a stannous fluoride and sodium fluoride toothpaste on erosion showed that the stannous fluoride toothpaste reduced erosion by 39% compared to the sodium fluoride toothpaste, which did not significantly reduce erosion compared to the water control (Huysmans et al. 2011).

1.13.5 Other Fluoride Compounds

There are other fluoride compounds that have been used in dentistry. Other monovalent compounds such as potassium and amine fluoride have also been tested as well as other polyvalent compounds such as aluminium, magnesium and amine fluoride. These have not been studied as extensively and their use in oral care products tends to be in conjunction with the more conventional fluorides. Wiegand et al. investigated the protective effect of titanium, amine and stannous fluoride against hydrochloric acid erosion. The authors' reported that all fluorides reduced calcium loss compared to a control by 58-67%, with amine fluoride (AmF) being the most effective and stannous fluoride being the least. Amine fluoride has the highest pH (pH 4.3), which might explain why it produced the least erosive tooth loss. However, it is not so simple. Titanium tetrafluoride, at pH 1.3, was lower than stannous fluoride (pH 2.6) but gave better protection, suggesting that the metal ion has an important role (Wiegand et al. 2014). Yu et al. investigated five fluoride compounds (TiF_4, NaF, AmF,ZnF_2 and SnF_2) at their native and buffered pH on *in vitro* enamel erosion, measuring the step height loss with contact profilometry (Yu et al. 2010). At the same buffered pH, these authors' reported that AmF

produced the least mean step height (MSH) (0.16µm ±0.30) compared to the others, with the highest being TiF_4 at 2.34µm ±0.38. Interestingly, even at the native pHs, AmF (pH 4.6) still produced the lowest MSH (0.17µm ±0.32) which was almost the same as the buffered value however the highest step height lost this time was for ZnF_2 at 2.38µm ±0.08.

Using calcium analysis and confocal microscopy to measure step height, Vieira et al. also investigated titanium tetrafluoride and amine fluoride (A. Vieira et al. 2005). They reported that at the same concentration, titanium tetrafluoride produced a similar mean step height (MSH) (8.29µm ±0.39) compared to amine fluoride (8.69µm ±0.66) and calcium release (approximately 0.4 µg/mm^2). However in this study the titanium tetrafluoride was formulated as a gel and the amine fluoride was a varnish, which may have influenced the results. The more viscous varnish may remain on the enamel longer, which increases contact time with the fluoride whereas the gel being aqueous may have faster clearance.

There are several ways in which fluoride can be delivered to teeth. These are by toothpaste, mouth rinses and varnishes in the form of soluble free-fluoride. The most commonly used methods are by toothpaste and mouth rinses. Varnishes and gels are generally applied by dental professionals and so are less widely used. Assessing the different delivery systems and formulations for their ability to protect against erosion is difficult and care must be taken. All contain a variety of ingredients for stabilisation, thickening etc. and abrasives. The interaction between all the different components and their effect on the anti-erosive effect is not fully understood.

1.13.6 Toothpaste and mouth rinses

The evidence to suggest that toothpastes and mouth rinses protect against erosion has mainly been derived from the laboratory (Levy et al. 2014; Ganss et al. 2013; Mathews et al. 2012). Toothpastes are limited in the EU to a concentration of around 1500 ppm or less, however

prescription toothpaste can be as high as 5000 ppm (Lippert 2013). Mouth rinses tend to be at the lower concentration (300ppm).

For a toothpaste to be effective in preventing erosive tooth wear, it has to be effective even after the abrasive brushing element. In 2013, Lippert suggested several reasons why the best way to deliver fluoride compounds (along with other active ingredients) would be through mouth rinses (Lippert 2013). Toothpastes once in the oral cavity need to be dispersed, ideally uniformly; this is achieved with the toothbrush and also mixing with saliva to form slurry. This slurry dilutes the toothpaste, increases the temperature and alters the pH. Also the toothpaste formulation contains many additional compounds, which can influence the bioavailability of the fluoride. These factors are highly variable and often unique to the individual as brushing techniques vary as do salivary flow and compositions. With rinses on the other hand, distribution is not as dependent on saliva, the interaction with the saliva is minimal and thus relatively higher doses and concentration of fluoride compared to tooth brushing can be delivered (Lippert 2013).

1.13.7 Varnish

Varnishes and gels allow higher concentrations of fluoride to be applied, for example Colgate Duraphat varnish has a sodium fluoride concentration of 22600ppm. A review of the literature on fluoride in erosion by Huysmans et al. showed that, in common with toothpastes and mouth rinses, the evidence suggests that varnishes can provide protection from erosion (Levy et al. 2012; Levy et al. 2014; A. Vieira et al. 2005; Huysmans et al. 2014). Whilst sodium fluoride varnishes were initially investigated in 1994 (Sorvari et al. 1994), TiF_4 was investigated 3 years later in 1997 (Büyükyilmaz et al. 1997) and was shown to be more effective with concentrations around 24500ppm. Vieira et al in a laboratory investigation into highly concentrated (>12500ppm) fluoride showed a significant effect on protection from erosion (A. Vieira et al. 2005). Austin also showed a similar response in another laboratory investigation

comparing high fluoride formulations (>5000ppm) of sodium fluoride and showed a dose response mechanism although the results were not linear indicating a more complex reaction than a simple increase in concentration of fluoride (Austin 2011).

Varnishes have also been investigated as a source of high fluoride. Vieira et al. observed reduced erosion in the presence of fluoride varnishes (A. Vieira et al. 2005). Varnishes may be able to provide protection against other forms of erosive tooth wear. Huysmans et al. investigated the abrasive element, acid immersion times and strength of acid and reported that varnishes were protective in an erosion-abrasion environment (Huysmans et al. 2014). Austin et al. also showed in a laboratory study that highly concentrated fluoride in the form of a varnish could protect against attrition (Austin et al. 2010).

1.13.8 Laboratory models

When used in laboratory investigations fluoride can be applied via slurry or directly by brushing. Brushing might be expected to reduce the protective effect of the fluoride. Ganss et al. investigated several toothpastes for their effect when applied as slurry or with abrasion and reported that for sodium and stannous fluoride, application as a slurry provided better protection than when applied with brushing (Ganss et al. 2011). This suggests that the effect of brushing can reduce the protection of the fluoride.

1.14 Model variables

1.14.1 Sample size calculation

When designing an experiment, often the first areas for consideration are the power and sample size. This is to ensure that a true statistical significance can be observed in an experiment. This is calculated based on previous published statistics or researchers experience in the particular field. A statistician should be consulted before starting the experiment. In a paper discussing methodology and models in erosion research, Shellis et al. suggested that

'sample sizes should be determined by appropriate power calculations'; this highlights the difficulties in this calculation as 'appropriate' will differ depending on the person calculating.

The main factor affecting the sample size calculation is the measurement outcome. The sensitivity and accuracy of the testing method will influence the sample size. Knoop microhardness change is not a very sensitive measurement system and it produces data that can be highly variable. In order to achieve adequate power a larger sample size would be needed. This contrasts with very sensitive testing methods, which produce data with a low standard deviation. The sample size could be small, as is the case for profilometry, as this technique can measure samples to a high resolution. For example, Hemingway et al. used profilometry as their main measurement technique and had a sample size of 8 per group (Hemingway et al. 2010) whereas Lussi et al. used Knoop microhardness as the main technique and had a sample size of 10 per group (Lussi et al. 2014).

Problems can arise when several measurement techniques are used in the same study as the author will have to decide which measure the sample size will be based upon, this could then have an adverse effect on calculating statistical differences for the other measurement technique. A way around this would be to select the maximum sample size among the measures.

1.14.2 Sample Preparation

Sample preparations for *in vitro* experiments are highly variable. There are several stages to preparing samples, which include tooth selection, grinding/polishing, embedding, creating reference/exposed enamel and storage of the prepared samples. The reporting of these variables and level of detail depends upon the author and can vary greatly.

For most profilometry and microhardness investigations the enamel or dentine sample needs to be flat as neither profilometry nor microhardness can be used to accurately measure curved surfaces. The disadvantage of polishing is that it removes the outer highly mineralised surface

of the enamel which may have a different influence on any erosive regime that the inner areas. Different researchers have reported different polishing protocols. The grinding and polishing is a diverse stage with a variety of materials available such as grinding stones, disks, paper and foil (Silicon carbide), diamond pads, films (diamond and aluminium oxide), polishing cloths and suspensions. Silicon carbide disks are most commonly used in the preparation of enamel. There is the US and the Federation of the European Producers of abrasives (FEPA) and both use different numbering systems for the same grain size.

Surfaces are usually ground to create a flat area and then polished to remove defects. The end stage varies, with some authors deciding to polish with disks to a grit number of 1200 (grain diameter 12µm) (Vieira et al. 2011; Wang et al. 2012) and others using diamond sprays (grain diameter 1µm) (Gonçalves et al. 2012; Levy et al. 2012; Zero et al. 2010). Polishing to different final grit sizes will probably produce surfaces with different baseline characteristics. Field et al. reported that the same sample preparation method (e.g. polishing procedure) performed on human, bovine and ovine enamel produced surfaces with significantly ($p<0.001$) different baseline characteristics of roughness and microhardness (Field et al. 2014). Although the study by Field et al. compared different enamel substrates, the polishing produced different quality of surfaces.

The polishing procedure depends upon different factors in the experimental design, such as, the measurement technique and erosive protocol. For profilometry and step height loss, high amounts of erosion are possible to measure even without polishing. Ganss et al. reported that erosive depth of at least 50µm could be measured on unpolished enamel surfaces. They also observed that polished enamel resulted in increased erosion compared to unpolished enamel. They attributed the increased susceptibility to erosion to the lower surface mineral content and the interconnecting pores on enamel nearing the enamel dentine junction (Ganss et al. 2000). However, in most cases, smaller changes are measured and so an extremely flat surface

is required. Polishing to 4000 grit silicon carbide disk or using an aluminium oxide paste can produce surfaces with a flatness tolerance of less than 0.1μm (from reference to centre or polished area (Attin et al. 2007)) or a surface with a baseline curvature of less than 0.05μm (Vieira et al. 2011). The different end points for polishing and ways to measure baseline characteristics differ and this inevitably leads to variations between studies but as long as the same procedure is followed within the study, this should not affect the outcome of the individual study.

In conclusion, the choice of polishing regime depends on the study design. Accurate measurement for profilometry requires optically flat surfaces but this deviates from the clinical situation. Curved natural surfaces are currently difficult to measure with step height so in laboratory studies polishing is necessary but will ultimately depend on the study design.

FEPA Standard	60	80		120		180	220			320			500		800	1000	1200	2400	4000
U.S Grain number	60	80	100	120	150	180	220		240		280	320		360	400		600	800	1000
Grain Diameter (µm)	**260**	**200**	**160**	**125**	**93**	**76**	**68**	**58**	**52**	**46**	**39**	**35**	**30**	**25**	**22**	**18**	**14**	**10**	**5**

Table 4 Table showing the Federation of the European Producers of abrasives and US standards for silicon carbide disks and the corresponding grain number

Table 4 shows the Federation of European Producers of Abrasives and US number allocations with the corresponding grain sizes of polishing discs. For the lower grit sizes, up to grit size 220, both the US and FEPA standards use the same numbers. However, after this the values differ between the FEPA and US. Quite often, the standard or sequence used is not reported in the methodology. No studies, to the author's knowledge, have investigated the effect of different end points of polishing on *in vitro* erosion. There may be an effect on the roughness particularly depending on the final grit sized used. This factor becomes important when studies use profilometry to measure step height or change in roughness, as the initial roughness of the surface will be determined by the polishing protocol (final grit size, method of polishing, time of polishing).

1.14.3 Tooth Type and Surface

When using human teeth, molars and premolars are the most commonly used because of their availability. The tooth surfaces that are most often reported are the buccal and palatal/lingual surfaces as they contact the acids present in food and drinks. The proximal surfaces, whilst appropriate for caries models, are not suitable for erosion models as they do not have the same contact with acids. The cusps on the occlusal surfaces of posterior teeth mean they are difficult to flatten without exposing dentine and are therefore too challenging to measure with profilometry or microhardness. The buccal and lingual surfaces of molars are easier to prepare for embedding and grinding/polishing as they have the thickest areas of enamel and so are more commonly used. It is known that enamel thickness and mineral content can differ between teeth and on different areas of the same tooth (Sabel et al. 2009; Wong et al. 2004) which has potential to influence the amount of erosion caused.

A study by Carvalho and Lussi investigated the effects of human molar and premolars on the buccal and lingual surfaces to varying enamel depths (200, 400 and 600µm from surface) on *in vitro* initial erosion and measured the surface microhardness and calcium release (Carvalho &

Lussi 2015). They reported no significant difference (p=0.370) for both measures between lingual and buccal surfaces but a significantly greater (p<0.05) softening in premolars compared to molars. There was no difference in microhardness loss as depth increased but there was a significant decrease in calcium release. This study shows that there is a difference in the susceptibility of different teeth and surfaces to erosion and that the type of measurement is important depending on the substrate.

The thickness of the sample will also vary and depend on the requirements of the experiment and the size of the tooth. A thickness of 2mm is common. Embedding samples, in acrylic or epoxy resin or similar materials, ensures that polishing produces a uniform surface.

A smear layer is formed on the enamel surface following any polishing procedure. The smear layer consists of polishing debris, firmly attached to the surface after polishing. Watari showed that polishing enamel to a 2000 grit level (FEPA), produced a smear layer of approximately 0.27µm (Watari 2005) and Sanches et al showed that this layer was soluble in acid (Sanches et al. 2009). The thickness and consistency of the smear layer are influenced by variations in the polishing procedure. Other authors have shown that the smear layer is present when bonding to dentine or enamel (Bortolotto et al. 2009). It is a consequence of any polishing procedure but what the effect has on any laboratory erosion model is unknown. Removal of the smear layer is relatively straightforward as it is a mineralised deposit and so soluble in acid. Retaining the smear layer has the potential to act as a barrier to any erosion investigation so it is important to understand what affect the retention or removal has on the outcome.

1.14.4 Storage

Enamel samples are normally stored in water, artificial saliva or in a remineralising solution in the fridge or at room temperature. Attin et al. investigated how the storage conditions of enamel after an erosive challenge influenced profilometrical measurements. The authors' concluded that step height measurement of enamel was not affected by storage in wet or dry

conditions or by de/rehydration (Attin et al. 2009). Storage in water would in theory hydrate the enamel and possibly influence the amount of erosion but length of time of storage may also be important. There are no studies, to the best of this author's knowledge that have investigated the effect of sample storage prior to an erosive challenge.

1.14.5 Erosion cycle

The choice of protocol for erosive cycles is important and affects the experimental outcome. All authors describe the basic information and include the length of the experiment, number of cycles, time of immersion and the amount of solution. More detailed information is often omitted, such as, the temperature, how often the solutions are changed, storage between cycles, flow and flow rate if applicable. There is no standard and universally agreed way to report this information and so protocols become difficult to decipher and can be misinterpreted.

1.14.6 Container

The shape, size and to a lesser extent the composition of the container used to erode enamel specimens *in vitro* is subject to variation. The volume of the container used for the acid is based on the shape/size of the specimen and amount of solution being used, which again is highly varied. The important factor is that the solution covers the samples. The containers could be glass (Eisenburger et al. 2004) or plastic (Eisenburger et al. 2001) and of varying size. The reporting of the containers varies, with some giving detail of (width, height, base, material) (Eisenburger et al. 2004) and others simply stating 'container' (Mohammed et al. 2013). The size and shape of the containers used in *in vitro* erosion might influence the flow of the solution and this is turn would affect the amount of erosion (Attin et al. 2012; Eisenburger & Addy 2003; Shellis et al. 2005; Wiegand, Stock, et al. 2007).

1.14.7 Position of sample

The position of a sample in an erosive solution may also be important. In most cases, the sample should be fully submersed in the solution and this can be achieved in two ways; either the sample is facing up and the surface of interest is facing out of the container or the surface is facing down into the container. In both cases the surface is fully covered with the solution. However, the flow of the liquid over the sample could be different. Paepegaey et al. investigated this aspect in an *in vitro* erosion investigation with orange juice. The authors' observed that suspending them upside down produced a lower step height loss but the difference was not significant (Paepegaey et al. 2013). Although the practicalities of suspending samples in the solution is more difficult than simple immersion, it raises the prospect that the position of the sample is important (Pretty et al. 2004; Elton et al. 2009; Ablal et al. 2009).

1.14.8 Agitation

Agitation of the erosive solution is important for *in vitro* experiments because clinically, an erosive solution is rarely static. Investigations on enamel (Eisenburger & Addy 2003; Shellis et al. 2005; Attin et al. 2012) and dentine sections (Wiegand et al. 2007) have shown that increasing the speed of the agitation increases erosion, probably by a replenishment of fresh ions leading to increased dissolution. Flow and flow rate of the solution maybe an important factor and increasing the flow rate should increase the amount of erosion (Eisenburger & Addy 2003; Shellis et al. 2005; Attin et al. 2012). Studies have reported no agitation (Scaramucci et al. 2011; Levy et al. 2012), a variety of flow rates using different commercial instruments (Attin et al. 2012) or bespoke (Eisenburger & Addy 2003; Shellis et al. 2005) machines aimed to replicate the mouth. But replicating the mouth is virtually impossible and a static response undervalues the effect of erosion and so some form of agitation should be considered. What is not known is how different systems might influence the amount of erosion.

1.14.9 Erosive Solution

The solution should cover the samples and the volume required depends on the number of samples per group, sample size and size of container. The total volume of the solution used is normally quoted but the volume of solution used per sample should also be included. For example, two studies using cola as the erosive agent had large differences in the amount of solution per sample. Choi et al. used 50mL per sample in their experiments (Choi et al. 2012) but Fujii et al. used 1.6mL per sample (Fujii et al. 2011). But on further examination a comparison between the studies is impossible as Choi et al. used atomic force microscopy (reporting step height) and scanning electron microscopy and Fujii et al. used contact profilometry (reporting surface roughness) and pH.

The range of volumes used can be as low as 1mL (Attin et al. 2012) to as high as 500mL (Shellis et al. 2005). Shellis et al. studied the effect of variable volumes of a 0.3% citric acid solution adjusted to pH 3.2 and observed that increasing the volume produced a linear increase on the amount of erosion (Shellis et al. 2005). The increase in volume means there is a greater supply of acid molecules and protons to attack the enamel surface. Many of these differences are predictable but if the authors do not describe precisely the environment under which the study is conducted comparison between studies is not possible.

The choice of which type of erosive solution used is dependent on the research question. The main factor is whether to use a pure acid or a commercially available product. The effect of fruit juices (Penniston et al. 2008; Gonçalves et al. 2012), candy (Wagoner et al. 2009), soft drinks and medications (Lussi et al. 2012) on *in vitro* erosion have all been extensively investigated. The most common dietary acids are citric and phosphoric acid and they have been studied extensively (West et al. 2000; Shellis et al. 2013; Barbour et al. 2003), however, clinically, these do not exist in isolation. Erosive foods and drinks contain a combination of acids in a variety of ratios. For example, coca cola contains citric acid and phosphoric in

approximately a 3:1 ratio (Featherstone & Lussi 2006). The interaction between different acids could alter their erosive potential.

Viscosity of the erosive solution has also been shown to affect the amount of erosion produced. Aykut-Yetkiner et al. investigated the effect of different viscosities of citric and phosphoric acid on enamel and observed that at a higher viscosity less erosion was formed by both acids (Aykut-Yetkiner et al. 2013). This is the only paper to the author's knowledge that compared the effect of viscosity on erosion and there is a need for further work. Investigating the effect of temperature and viscosity together also needs to be evaluated as viscosity is also influenced by temperature. A study by Kestin et al. reported the outcome on bovine enamel between viscosity and temperature but at 20°C (Kestin et al. 1978). What the effect is on human enamel is unknown (Attin et al. 2007).

1.14.10 Cycles

The description of the detail of the erosive cycles varies. For example, Stenhagen et al. reported that enamel samples were immersed in 15mL of hydrochloric acid, under gentle agitation for 12 minutes (Stenhagen et al. 2012). Whereas, Gonçalves et al. reported a more detailed description; one cycle consisted of immersion in 25mL of grape juice for 10 minutes at room temperature under agitation with a pump set at 3600rpm. There were 4 cycles per day, with 3-hour intervals. The samples were stored in artificial saliva between cycles and new solutions were used for each cycle. The experiment lasted 15 days (Gonçalves et al. 2012). Generally a longer cycle will produce a greater amount of erosion. However, Stenhagen et al. observed that a strong acid resulted in more erosion with a shorter time (Stenhagen et al. 2012).

Other methods such as cycling between demineralisation and remineralisation are typically employed in caries models (Lynch 2006). This type of cycling can contribute to making *in vitro* studies clinically relevant. This adds further variables that need to be considered, Vieira et al.

investigated the effect of fluoride dose response in pH-cycling models and varied the time of demineralisation, composition of the de and remineralisation solutions, frequency and time of application of experimental solutions and the pH-cycling duration (A Vieira et al. 2005). The role of pH-cycling and standard cycling is to provide more quantitative data to inform better designed clinical studies (Roberts 1995; Buzalaf et al. 2010). Whilst pH-cycling models are mainly used for caries models it has been adapted and a de/remineralisation process has also been applied to investigate erosion.

1.14.11 Immersion time in an acid solution

It has been shown that increasing immersion time in an acidic solution increases the amount of erosion caused *in vitro* (Torres et al. 2010; West et al. 2000; Stenhagen et al. 2010; White et al. 2010). The immersion time depends partly on how much erosion the researcher wants to create and this relates to the hypothesis but also to the method of measurement. If the hypothesis involves an early enamel erosive lesion then the immersion time tends to be less than a few minutes. However, if a larger amount of erosion is required, then the time increases. Most studies, consider a clinically important value of between 5 and 10 minutes, as this is the time it can take to consume a drink (Bartlett et al. 2011). Erosion times can be as low as 2 seconds (White et al. 2010) in an early erosion model or as high as 15 hours (5 minutes per day, 3 times, with 4 hour remineralisation between immersion, for 60 days) (Torres et al. 2010).

Clinically, the amount of time an acid solution will be in contact with the tooth surface and therefore how much erosion is caused, is variable and dependent upon the drinking habits of the individual (Bartlett et al. 2011). If a drink is swallowed immediately then the contact time with the enamel surface will be minimal. However, if the drink is swilled around in the mouth or consumed over a long period of time, then this time will be increased. It becomes difficult to accurately simulate acid exposure times *in vitro* meaning a judgement has to be made for an

immersion time based on the research question, erosive solution and the measurement outcome.

The number of cycles (that is, immersion in the acid followed by removal and subsequent re-immersion) used in *in vitro* studies is similar to the immersion time and depends on how much erosion is desired. There is no agreed standard for how many cycles are relevant and cycles can vary from 1 to as high as 180 (Torres et al. 2010). It is a compromise between accurately simulating the clinical situation and how much time/resources are available.

Rinsing between the cycles is another variable. The aim of rinsing between or at the end of the cycle is to remove any of the experimental solution (acid, toothpaste, saliva) from the surface or, if after an erosive challenge to stop the erosive process. Typically this is done with distilled or deionised water. However, the duration and method can vary and it is unknown whether this would have an effect on the measurement outcome. Barbour et al. showed that rinsing enamel with distilled water between immersion in distilled water produced no measurable softening with nanoindentation (Barbour et al. 2003). Samples can be rinsed in deionised water (Favretto et al. 2013), distilled water (Barbour et al.) or tap water (Vieira et al. 2011). Reporting the rinsing also varies, with some reporting the time, varying from 5 seconds (Sun et al. 2014) to 1 minute (Vieira et al. 2011), the volume (Barbour et al. 2003) or reporting the event 'rinsed with tap/distilled/deionised water (Carvalho & Lussi 2014; Favretto et al. 2013). As with all of the variables, more detail is better as this allows for better comparisons and re-creation of the experiment conditions.

1.14.12 Temperature

There are two studies showing that the temperature of an erosive solution affects the amount of erosion produced. Eisenburger et al. and West et al. investigated the effect of varying temperatures of citric acid on enamel and both observed increased erosion at higher temperatures. Direct comparison of the studies is difficult as both used different protocols.

Eisenburger et al. used 0.3% citric acid adjusted to pH 3.2, agitated at 270rpm and immersed the samples for 2 hours. Whereas West et al. used 0.3% citric acid with an unadjusted pH, with gentle agitation and immersed the samples for 10 minutes (West et al. 2000; Eisenburger & Addy 2003). Both studies observed that erosion increased with increasing temperature.

1.14.13 Abrasion

Most abrasion studies on enamel tend to follow an erosive challenge as this simulates eating/drinking followed by toothbrush abrasion. A review of the literature in 2003 showed that abrasion alone either from the toothbrush or the toothpaste without an erosive challenge caused almost no surface loss (Addy & Hunter 2003). The main variables associated with abrasion were; machine, toothbrush, time, force and toothpaste/mouth rinse which are discussed in detail below.

1.14.14 Automatic Brushing Machine

Automatic brushing machines allow standardisation of the abrasion regime, controlling the brushing frequency, force, time and movement. These machines can hold manual and electric toothbrushes and can be commercially available, such as the DentaGen V.1.50 Syndicad or Tooth brushing machine ZM-3 used by (Ganss et al. 2012). Other authors report custom made devices (Levy et al. 2012). The standard movement of the brush head is in a linear motion (back and forth) however another machine ZM-3 uses a zigzag motion and the latest model can create a variety of motions such as a linear motion in the x or y axis and a circular motion (Ganss et al. 2012). To the author's knowledge, there have been no studies comparing different automatic brushing machines.

1.14.15 Toothbrush

Toothbrushes vary in bristle hardness, pattern or whether they are manual or automatic. Generally a manual toothbrush is used for laboratory investigations. Voronets et al. reported the effect of toothbrush type on abrasion and provided a very detailed description of the

toothbrush including, filament composition, thickness and length (Voronets et al. 2008). Most studies quote the toothbrush type and brand. Although there is not a universal reference toothbrush for abrasion studies, the American Dental Association advise a reference toothbrush, Oral B P40 (flat bristle field 27mm x 10mm, bristle diameter 0.2mm). Consequently, some authors tend to use this brush (Ganss et al. 2012).

The time, force and frequency of brushing will increase the amount of wear (Addy & Hunter 2003). Wiegand et al. showed that tooth brush abrasion with a force of 4.5N and toothpaste with RDA =77 had very little effect on enamel wear (Wiegand et al. 2007). Again there was variation in the application of force. Ganss et al. used a mean value of 2.3N (Ganss et al. 2009) whereas Wiegand et al. used 1.6N (Wiegand et al. 2012). The combined effect of abrasion and erosion remains a complex and little understood interaction. Brushing forces can be expressed in Grams or Newton's. Most studies use a force between 2-3N as this replicates the mean load applied during brushing with a manual toothbrush (Ganss et al. 2009; Wiegand & Attin 2011). However, higher forces can be used. Wiegand et al. also used a force of 4.5N (Wiegand et al. 2007).

1.14.16 Time

The frequency and timing of the brushstrokes is another varied area. Some clinical studies have reported brushing time to between 30-90s, which is equivalent to 300-400 brushstrokes (depending on the brushing machine) (Lussi & Jaeggi 2006; Wiegand & Attin 2011). Ganss et al. used 38 brush strokes (Ganss et al. 2012) but Voronets et al. used 590 brush strokes (Voronets & Lussi 2010). Ideally, the total number of brush strokes should be quoted as it allows for better comparison between studies. The total number of brushing strokes in the mouth during tooth brushing may be high but the number of strokes each tooth receives is generally only a few. The conflict is which to choose. Another factor is the replacement of the toothbrush; higher force, toothpaste abrasivity and frequency might lead to softening and fraying of the

toothbrush head. This should affect the performance of the brush and therefore the amount of abrasion and so it may require replacement more frequently. These factors have not been investigated.

1.14.17 Toothpaste/Mouthwash

The choice of toothpaste can be separated into two main categories, fluoridated and non-fluoridated, with the latter producing more wear *in vitro* (Wiegand & Attin 2011). Additionally the abrasivity of the toothpaste is important, however this is rarely mentioned even though it has been shown to have an effect on the amount of abrasion caused (Wiegand et al. 2009). It is usually used as a toothpaste slurry, with toothpaste to artificial saliva (Voronets et al. 2008) or water (O'Sullivan & Curzon 2000) in the ratio of 1:3 or even higher at 1:10 (Lussi 2006). This replicates dilution of toothpaste when used clinically.

Overall the detail and description of erosion models varies considerably between different studies. In some cases the choice of method may have a significant effect on the result and so the implication of the study.

1.15 Measurement of Tooth Wear *in vitro*

Surface measurement of the enamel is a common way to assess the impact of the erosive wear. This can be done quantitatively through profilometry and atomic force microscopy or qualitatively through scanning electron microscopy and atomic force microscopy.

1.15.1 Profilometry

Non-contact profilometry uses a light source to measure the change in surface profile, whereas contact profilometry uses a stylus head that moves over the surface. The surface for both techniques should be polished to provide a flat surface and to optimise the accuracy and sensitivity in the measurements. Non-contact surface confocal profilometry uses an optical stylus sensor, producing a laser light, with a small spot size (e.g. 7µm), a spectrometer and a

highly precise motion controller. The system is controlled by a computer with a manufacturer's programme.

Figure 8 shows a schematic of the measurement principles of the confocal sensor. A polychromatic white light is focused, via a fibre optic cable, onto a surface using a chromatic lens in the sensor head that disperses the white light into monochromatic light. A factory calibration sets each wavelength at a certain deviation, which relates to a specific distance from the target. Only the wavelength that is exactly focused on the target is used for a measurement. The light reflected back from a surface returns through the probe via a beam splitter and is then passed through a confocal aperture onto a spectrometer. This analyses the spectral changes of the reflected light, from which distances and heights of a surface can be accurately measured. Data points are collected as the sensor moves across the surface measuring the reflection of the laser light via the confocal method (Schlueter et al. 2011).

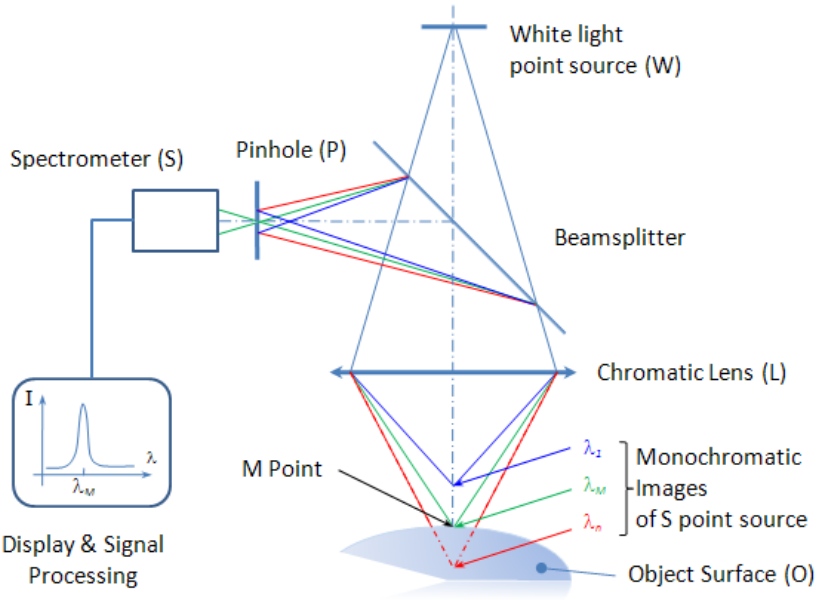

Figure 8 Schematic of the measurement principle for the confocal white light sensor

The raw data can be exported and analysed using computer software. Profilometers are usually packaged with their own bespoke software for analysing the data e.g. Proscan 2000

(proscan application software v2.0.17), MicroProf (Mark III) and Taicaan XYRIS (Boddies). These usually contain a set of features that can extract information such as surface profile (step height), roughness or the texture of the surface.

Step height change requires a reference area that has not been affected by the erosive solution in order for a comparison to be made with the eroded area, Figure 9. The height from the reference area to the bottom of the worn area relates to the amount of erosion and is called the step height. This can be a single line profile, a mean value over the length of the wear scar or the volume. The instruments commonly have a number of additional measurement functions including roughness (surface roughness average or root mean square) (Field et al. 2010).

1.15.1.1 Step height loss

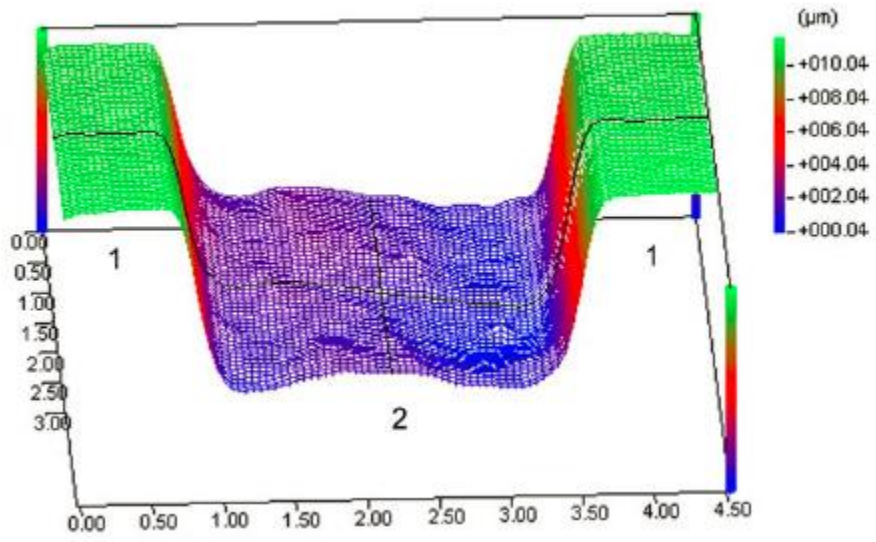

Figure 9 Image showing a step height loss measured by non-contact laser profilometry, showing the unaffected reference areas either side of the worn central area from (Vieira et al. 2006)

Commercial software can be used to analyse the data. Bespoke macros from open source programs such as ImageJ can be written to allow batch processing of data. MountainsMap® is

a surface metrology software that can analyse an array of data formats in accordance with the latest ISO standards.

Non-contact surface profilometry has been reported to measure step height change to quantify the loss of dental tissue compared to a reference area (Bartlett et al. 1997; Azzopardi et al. 2000). Over any large surface the regularity of a sample changes. Although this can be adjusted by changing the baseline curvature and data outside a pre-determined range the machines are more accurate over smaller areas (Vieira et al. 2011).The advantage of non-contact surface profilometry is the data can be collected without any damage to the surface of the sample and with a higher resolution compared to a contacting profilometer (Attin 2006).

Non-contact surface profilometry cannot measure the sub-surface area, demineralisation or surface softening of eroded samples. This puts it at a disadvantage compared to techniques such as transverse microradiography. The scanning process is also influenced by colour and transparency of the specimen. Authors have observed that if material absorbs colour at the same wavelength as the laser, then the surface cannot be accurately scanned (Rodriguez & Bartlett 2010; DeLong et al. 2001). The scanned area needs to be controlled as 'overshots' at the sharp edges or at the bottom of the sharp grooves may result in artefacts, over measuring of the surface which results in phenomena that is not there (Whitehead et al. 1999).

Using a profilometer to assess samples in erosion research has many uses. Any surface metrology phenomenon can be investigated, however for erosion research the main assessment is step height change. This measures change in height of an exposed enamel surface in comparison to a reference area. A single scan across the surface by the profilometer produces a 'profile' of the surface. If this is repeated across the entire sample then a 3D representation of the surface is produced and allows a variety of ways for step height change calculation. A single line step height identifies a single mid-point profile and is used if scanning the entire surface is not possible (Attin et al. 2009; Hooper et al. 2007; West et al. 2003). This

was typically used in the early stages of profilometry and erosion research. If several single line step height changes are taken across the surface then a 'mean step height change' can be calculated and might be considered to be more representative of the entire sample (Magalhães et al. 2010). However the true meaning of a mean step height change is one where every profile of the surfaces is calculated along the whole length of the wear scar (Rodriguez & Bartlett 2010). There are ISO standards available that often come pre-programmed with commercial software however authors can also extract the data in different methods manually. However, although each method presents the data in a different way the underlying theme or comparisons should in theory not vary significantly.

In a study undertaken at three laboratories at different locations, the same sample was scanned by a non-contacting profilometer, a white light source and a microscope. The absolute data output varied between each machine but the statistical differences between the different samples remained consistent. Therefore if samples are measured with different machines using different calculations of step height, the data output although different, should have similar comparisons provided the machine is used within its own accuracy and reliability (Paepegaey et al. 2013). The implications from this study are that it does not matter what type of instrument is used to scan the samples for step height loss (Non-contact white light profilometer, contact profilometer and confocal laser scanning microscope) as long as the technique and instrument are kept constant then the overall results will be the same.

Contact profilometry differs from non-contact profilometry in that is uses a metal or diamond stylus (approximately 20μm wide), loaded with a force (typically a few millinewtons), to physically scan the surface by moving the stylus over the specimen. The resolution can be less than some non-contacting profilometers as the spot size on the non-contact profilometer can be as small as 2μm. The main parameters that affect measurement are the force applied to the stylus and the speed over which it travels across the surface. As the stylus physically touches

the enamel it may interact with the surface and could penetrate the outermost demineralised layer which is susceptible to mechanical forces potentially overestimating the erosion depth (Schlueter et al. 2011; Attin & Wegehaupt 2014). The scratching of the surface is not ideal as the alteration makes repeat measurement of the same area difficult. However, scratches may be viewed via atomic force microscopy and the depth can be quantitatively measured (Beyer et al. 2011; Beyer et al. 2012). This can be time consuming and expensive. However, unlike non-contact profilometry the contact stylus is not affected by water so it has the potential to measure wet surfaces or samples. Attin et al. recommended using contact profilometry for dentine samples as dentine shrinks when dried. This avoids the data being affected by the ambient conditions (Attin et al. 2009).

The accuracy and reliability of a profilometer is affected by many factors. With such high precision and resolution, regular maintenance must be carried out to ensure the data is accurate and reliable. Noise errors, such as vibrations and temperature variations, can affect the operating of the machine and both these factors need to be considered when installing the instruments (Jcgm 2008). The effect of vibrations can be minimised by using shock absorbing bases. The effect of heat on the sensitive components controlling movement can be minimised by using materials that are not affected by heat expansion and contraction by placing in a temperature controlled room. A study by Austin investigated the accuracy of a non-contact profilometer, using a step height reference standard (Taylor-Hobson, Leicester, UK), to assess deviation of the profilometer from the standard. The author reported that the profilometer was accurate to within 0.02µm and that as the depth of the step height increased, the accuracy increased (Austin 2011).

1.15.2 Scanning Electron Microscopy (SEM)

Scanning electron microscopy uses an electron beam to produce an image of a surface. The samples must be small enough to fit in the instruments vacuum chamber and the surface,

which can be polished, unpolished or native, must be gold sputtered. This technique produces an image with a high resolution and depth of field. However, it is expensive and the high vacuum can cause tissue shrinkage and loss of fluid during sample preparation. This may alter the surface and produce drying artefacts (Attin 2006). Environmental SEM allows imaging of wet samples, without a metal coating and in a low vacuum and so less sample preparation is required; resulting in potentially less damage and allowing multiple images to be easily obtained. This is a qualitative method and so changes to the surface must be estimated (Torres et al. 2010; Magalhães et al. 2010). The technique can be used to study etching patterns (Li et al. 2013; Hobson et al. 2002) or to visualise the effects of a protective product on the surface compared to a reference area (Lombardini et al. 2013).

1.15.3 Atomic Force Microscopy (AFM)

Atomic force microscopes are part of the larger group called scanning tunnelling microscopes. They can provide even higher resolution images compared to the SEM microscopes, down to the molecular level. This is achieved as the probe used to analyse the surface has a radius of just a few nanometres. AFM can be used to measure differences in height of up to one atom and also surface roughness. It can be used with and without a vacuum, in air or in liquid, which means the possibilities of artefacts are reduced. Due to its high resolution, only small areas can be scanned (approximately 0.5mm x 0.5mm), which can be time consuming (approximately 60 minutes) and several scans must be done to find a surface that represents the majority of the sample (Barbour & Rees 2004).

1.15.4 Hardness Testing

Measuring the hardness of an enamel surface before and after an experimental treatment is a relatively quick and convenient method to assess surface change. Most commonly, microhardness is used, however nanoindentation can also be used which is more sensitive. Both techniques involve the use of a diamond tip that is pressed onto the surface at a given

load and for a certain time. Both also, require a flat, highly polished surface for accurate measurements.

1.15.5 Microhardness

Microhardness has been used to investigate the effects of early erosion *in vitro* (Schlueter et al. 2011). The hardware consists of an indentation machine, which includes a stage, diamond indenter, variable loads and a microscope to view the sample. The time and load applied are controlled via the machines software. Typically a Knoop or Vickers diamond indenter is used to create an indent in the sample. The Knoop diamond-head leaves a rhomboid indent and Vickers, a tetra-pyramidal indent. A Knoop diamond is preferred it has a shallower penetration of the surface (1/30.5th of its length) compared to Vickers (1/7th of its length) and therefore better reflects the property of the outer most layer of an erosive lesion and less likely to be affected by the underlying structure (Schlueter et al. 2011). Using the American Society for the Testing of Materials (ASTM) E384 standard, the Knoop hardness (H_K) is obtained by optical measurement of the length of the long diagonal of the indent and putting this value into the equation shown in Figure 10.

Microhardness is a simple piece of equipment to analyse the surface and give information about its hardness. In erosion research, the main use has been to compare the change in hardness of the surface of enamel or dentine after an experimental procedure to a reference area. Researchers have also adapted the technique to calculate microhardness recovery or in the case of abrasion studies, to measure the depth of the indent and relate that to the amount of wear produced.

a)

$$H_K = P / (C_p\, d^2)$$

b)

$$\%SMR = 100[(Le1 - Lr)/(Le1 - Lb)]$$

c)

$$\%RER = 100[(Le1 - Le2)/(Le1 - Lb)]$$

Figure 10 a)Equation to calculate microhardness where P is the test load (gf), C_p is the indenter constant and d is the diagonal length (µm) b) surface microhardness recovery and c) relative erosion resistance, where Lb is length at baseline (µm), Le1 is length after first erosion, Lr is length after remineralisation and Le2 is length after second erosion

Figure 11 shows a calculation for microhardness; where P is the test load (gf), C_p is the indenter constant and d is the diagonal length (µm). Typically, a load between 50-100g and a dwell time of 15 seconds is used to indent the surface and the process repeated 3-6 times to give an average value (H_K) (Voronets et al. 2008; Zero et al. 2010; Scaramucci et al. 2011; Gonçalves et al. 2012; Wang et al. 2012).

Hara et al. reported the 'percent surface microhardness recovery' and after a further erosive challenge they calculated the 'percent relative erosion resistance' but used the indention length and not the hardness value (Hara et al. 2009). An average length was calculated from five indentations, 100µm apart, using a force of 50g and a dwell time of 15 seconds. 'Percent surface microhardness recovery' records a measurement at baseline (Lb), another after the first erosive challenge (Le1) and finally after remineralisation (Lr); Figure 10b. 'Relative erosion resistance', is calculated from these four measurements, one at baseline (Lb), one after the first erosive challenge (Le1), one after remineralisation (Lr) and one after the second erosive challenge (Le2), and then the value calculated using the formula (Figure 10c). Higher values represent greater recovery and therefore greater remineralisation potential or resistance to erosion. This technique is applicable to those models involving a remineralisation phase and although this is reported by a respected group of researchers, the microhardness machines

were not designed to measure samples where the indents were themselves affected by a challenge, which in this case was an acid.

Some research groups have used indentations to measure loss of enamel from abrasion. The volume or length of the indent is measured before and after abrasion and so provides an objective measurement of abrasive wear, assuming that the indentation is not altered by any other form of wear (Carvalho & Lussi 2014; Attin 2006; Jaeggi & Lussi 1999; Joiner et al. 2004). However, the technique is inappropriate for erosion experiments as the acid may erode the reference area around the top of the indent as well as the bottom of the indent (Attin T 2006; Schlueter et al. 2011). Microhardness data can also be used as quality control of polished enamel samples using a pre-determined value between H_K 300-370. Samples outside this range are discarded (Gonçalves et al. 2012). The main advantage of microhardness is that it is a quick and relatively low cost method to obtain accurate data on the surface properties during early erosion. Hardness values could be influenced by changes in the enamel surface if the indenter presses beyond the demineralised zone (Tabor 1986). For accurate measurement a flat highly polished surface is needed so that the indention edges can be clearly seen, this limits its use on natural tooth surfaces and makes measurement of eroded enamel surfaces difficult. Although in the early years of erosion research microhardness was used for early and late erosive lesions, the general consensus today, is that its most appropriate use is for the early lesion where the surface remains intact but demineralisation has occurred.

The accuracy and reliability of microhardness is dependent on several factors, such as sample flatness, calibration of the weights, timing mechanism and operator experience. To measure the accuracy, a reference block of known hardness is tested periodically to measure the deviation in the machine. The surface of such a block is highly polished and flat to provide the ideal surface for microhardness testing. Austin assessed the accuracy of his Leitz-Wetzlar microhardness machine and found it to be ±39.33HK from the true value (Austin 2011).

1.15.6 Nanoindentation

Nanoindentation is another form of surface hardness testing. It typically uses a diamond Berkovic tip, with a press load of a few milliNewtons producing a shallow indentation of 150-500nm (Finke et al. 2000). It has been shown to be sensitive for the assessment of the early stages of enamel dissolution *in vitro* (Barbour et al. 2003) and can provide nanomechanical properties such as Youngs modulus of elasticity, hardness, fracture toughness, time-dependent creep, plastic and elastic energy (Oliver & Pharr 1992). It can also be used in conjunction with an atomic force microscope to give a resolution in the vertical axis of 0.2nm (Jandt 2001). Elastic modulus has been shown to be more sensitive to the presence of underlying enamel compared to microhardness (Barbour et al. 2003) and therefore it is suggested to be useful for erosion studies (Barbour & Rees 2004). It has been shown to be extremely sensitive to very early stages of erosion. Barbour et al. found a significant change in enamel hardness after a 30 second exposure to citric acid 19.1mM, pH 3.3 (Barbour et al. 2005).

1.15.7 Elemental Analysis

This measures the release of minerals ions, such as calcium, phosphate and other minor constituents (fluoride and magnesium) from teeth as they pass into the solution. This change in concentration can be utilised to represent erosion. This can be achieved by several methods including atomic absorption spectroscopy, ion-selective electrodes and more recently, inductively coupled plasma mass spectroscopy. Elemental analysis of the surface of the enamel can be achieved by scanning electron microscopy – energy dispersive x-ray spectroscopy.

1.15.8 Inductively coupled plasma-Mass Spectrometry

Inductively coupled plasma – mass spectrometry (ICP-MS) is an established method in the chemical sciences to analyse very small concentrations of elements by processing the sample with inductively coupled plasma and then analysing with a mass spectrometer. However its use in dental research is limited with only 3 studies reporting it for elemental analysis; with

Carpenter et al. and Khambe et al. using it to analyse mineral content in saliva and Mita et al. using it to analyse mineral content from the erosive solution (Carpenter et al. 2014; Mita et al. 2013; Khambe et al. 2014).

1.15.9 SEM-Energy-dispersive X-ray spectroscopy (SEM-EDX)

SEM-EDX uses energy-dispersive x-ray spectroscopy to provide data on the composition of a surface. It is used to determine the composition of a deposition on a surface from a product (Schlueter et al. 2009; Ganss et al. 2010; Wiegand et al. 2009). It can be used to analyse the elemental composition on both sound and eroded enamel. Elements such as calcium, phosphorus, oxygen and carbon can be isolated on such spectra and their quantities analysed.

1.15.10 Ion-selective electrode

Ion-selective electrodes can be used to measure the concentration of calcium, fluoride, sodium and potassium. Typically, change in calcium concentration is used to measure erosion and fluoride electrodes are used to assess the uptake or release of fluoride from a sample. Parker et al. used an ion selective electrode and inductively coupled-plasma optical emission spectroscopy to measure calcium release and observed that both produced similar readings with a difference of 2ppm between them (Parker et al. 2014). Two papers published in 2014 used inductively coupled-plasma optical emission spectroscopy (Mohammed et al. 2014) and atomic absorption spectroscopy (Wiegand et al. 2014) to measure calcium release instead of an ion probe. In 2010, McGeouch et al. reported that scanning electrochemical microscopy and a 'moving bound finite element model', could analyse localised acid-induced dissolution by measuring the size and shape of the etch feature on bovine enamel (McGeouch et al. 2010). In 2014, Parker et al. used this technique for measuring the efficacy of calcium silicate for repair of the enamel (Parker et al. 2014). Scanning electrochemical microscopy uses an ultra-microelectrode that enables multiple controlled acid challenges to be targeted onto the

surface of enamel and the subsequent minute (approximately <5µm) etch pattern (or pit) is analysed by white light interferometry.

Ion electrodes are considered useful as they can be relatively quick and easy to use and the apparatus can be easily accessible in a standard laboratory. The disadvantages are that samples cannot be reused as solutions (e.g. total ionic strength adjustment buffer) need to be added to help the electrode measure the element in question more accurately. This adds further potential errors to the procedure.

1.15.11 Atomic absorption spectroscopy (AAS)

AAS is a method to quantitatively analyse the concentration of single elements at a time in a solution by changes to the colour. It is reliable and sensitive when measuring calcium (Schlueter et al. 2011) and can analyse small volumes (10µL) and concentrations (12.4µmol/L) of calcium (Attin et al. 2005) and between 1.9-9.0µmol/L of phosphate (Attin et al. 2005). However, it cannot be used in the presence of saliva or a smear layer as it would be affected by other minerals in the saliva (Schlueter et al. 2011).

1.15.12 Transverse microradiography (TMR)

Transverse microradiography measures the attenuation of a nickel filtered copper Kα-line X-ray irradiation transmitted through dental hard tissue, which allows the quantification of mineral loss to be obtained (Attin 2006). But the sample preparation is destructive. The samples are prepared in thin sections (50-200µm) and mounted onto a micro-radiographic plate (Attin 2006). Advantageously, this technique can simultaneously determine surface loss and show sub-surface demineralisation, which is a unique feature. There is a strong correlation between TMR and profilometry for calculation of mineral loss (Hall et al. 1997). But unlike profilometry, sub surface features can be explored.

1.16 Summary

Erosion is a common and complex process involving the interaction of acids, concentration, time and the influence of other wear processes. Past research has focused on the sources of acid such as; citric (Barbour et al. 2003), phosphoric (Aykut-Yetkiner et al. 2013), hydrochloric acid (Attin et al. 2012) or commercial products (Lussi et al. 2012). The effects of pH, acid, calcium/phosphate concentration, titratable acidity, buffering capacity and chelation have been investigated and all observed to influence the progression of erosion. Protection from erosion has also been studied, either by investigating the natural protection found with saliva (Ten Cate 2000; Featherstone & Lussi 2006) or by fluoride containing products (Lippert 2013), whereby the mode of action in how it protects against erosion remains debatable. However, most of this research has been derived from laboratory work.

The *in vitro* investigation of erosion and abrasion is important as it is often the first stage in exploring and understanding a problem. It also has the advantages of being relatively low cost and allowing greater control of variables. The main disadvantage is that any model will not replicate the human mouth but it remains the most convenient method to assess the action and interaction of the individual components involved with the erosive process. But variations in the study design, models variables and measurement techniques can influence the results and may compromise the conclusions made for a study.

The main finding of the literature review was that there is variation in protocols and model variables that might influence the data output. For example, although, bovine and human enamel are similar their structure and chemical composition differs (Yassen et al. 2011). Whilst bovine enamel is more conveniently sourced and larger than human enamel, creating a flat surface over a larger area creates different challenges. The choice of which substrate is selected is subjective but whatever choice is made data obtained can be utilised but with the appropriate provisions. Furthermore, the type of tooth and the surface may also influence the

outcome as the anatomy and orientation of the crystals changes in different parts of the same tooth. Other preparation variables that might influence the outcome are polishing, cleaning and storage of the samples. The effect of different study conditions may influence the conclusions and therefore they need evaluating. Another influence is the measurement system. Profilometry necessitates that the surfaces are flat and so they require polishing. Some researchers use high grain size grit diameters e.g. 12μm (Vieira et al. 2011) whereas others use lower ones e.g. 1μm (Zero et al. 2010). These choices are often made to maximise the accuracy and reliability of different measuring systems but may also affect the outcome.

If more than one measurement technique is used a dilemma occurs as typically one will be optimised rather than the other. Commonly both profilometry and microhardness testing are used in the same study (Stenhagen et al. 2010; Yamashita et al. 2013; Scaramucci et al. 2011) but profilometry requires greater depth and this conversely means that microhardness measurements are less accurate.

There may be other variation influencing the outcome of erosive models, such as, the size of the container, position of sample, immersion time and temperature. These fundamental aspects will affect the thermodynamics of a study. The addition of abrasion creates even greater complication. There is variation in human brushing habits (Ganss et al. 2009) which make it difficult to translate to the *in vitro* situation. For example, studies show a variety in force of brushing ranging from, 1.6N (Wiegand et al. 2012) to higher values of 4.5N (Wiegand, Kowing, et al. 2007).

The effect of the different protocols and variables on the study outcome has been given insufficient attention. If these are investigated and better understood, it could allow for more accurate comparisons between studies.

General aims of chapters 3, 4, 5 and 6

The aim of Chapter 3 was to investigate the effects of concentration and immersion times for common acids associated with erosion using changes in step height measurement and surface microhardness. It also investigated the repeatability of three mapping programmes.

Null Hypotheses tested:

- There is no consistency between different mapping programs when measuring the loss of enamel
- Immersion time, concentration and abrasion do not influence the amount of erosive wear

The aim of Chapter 4 was to investigate the effects of different *in vitro* model variables such as; tooth surface/type, ultra-sonication, storage of samples, mode and speed of agitation, rinsing, volume of acid and position of sample in the solution on the measureable outcome of step height and microhardness change.

Null Hypotheses tested:

- Changes to the following model design do not affect erosive wear
    - Tooth surface (Buccal/lingual) or type (molar/premolar) does not affect the step height or microhardness change
    - Ultra-sonicating after polishing does not affect the step height or microhardness change
    - Storing samples in deionised water for 1 or 24 hours prior to erosion does not affect the step height or microhardness change
    - Using Orbital, Gyro and See-saw agitation at 30, 40, 60 and 70rpm does not affect the step height or microhardness change

- Rinsing the samples with a spray bottle or a container between erosive cycles does not affect the step height or microhardness change
- Increasing the volume of acid from 80 to 100mL does not affect the step height or microhardness change
- Facing the enamel surface 'up' or 'down' in the solution does not affect the step height or microhardness change

The aim of Chapter 5 was to investigate the effect of sodium and stannous fluoride solutions on *in vitro* erosion.

Null Hypotheses tested:

- There is no difference in erosion with the application of sodium or stannous fluoride
- There is no difference in erosion when applying fluoride before or after an erosive challenge

The aim of Chapter 6 was to investigate a dose response effect of sodium and stannous fluoride on *in vitro* erosion using the protocols and knowledge developed from the previous Chapters.

Null Hypotheses tested:

- Increasing concentrations of sodium and stannous fluoride does not affect erosion when assessed using step height and microhardness

1.17 Central research hypothesis

Do model conditions affect the erosion or erosion-abrasion?

Using an *in vitro* model to investigate the effect of; type of fluoride, concentration of fluoride and timing of application.

Chapter 2. General Materials and Methods

This chapter reports the common materials and methods used in the thesis. It includes sample and solution preparation and cycling models for erosion and erosion-abrasion. It also describes the techniques for non-contact profilometry, microhardness and pH measurement.

2.1 Sample Preparation

2.1.1 Tooth Collection

Ethical approval for tooth collection was obtained through the National Research Ethics Service (NRES) London – Bloomsbury and Guys and St Thomas' research and development (REC REF: 12/LO/1836). The teeth were collected from floor 23 of Guy's hospital after gaining consent from the patient (see section for the patient information sheet and section for the consent form). After extraction, the teeth were disinfected and stored in sodium hypochlorite solution, for at least 72 hours.

2.1.2 Tooth Sectioning

Sectioning was performed using a circular saw (Buehler Isomet 1000 precision saw with an Extex diamond wafering blade) at a speed of 600rpm with a force of 1.0N using previously developed protocols (Austin 2011). Enamel samples were embedded in a copper tube filled with impression compound (Impression compound, Kerr, Green, type 1), Figure 11. Firstly, the root was removed from below the cementoenamel junction and then the buccal and lingual surfaces sectioned, with the cut starting at the cusp. All enamel sections were stored dry.

Figure 11 Image of tooth embedded in copper tube filled with green impression compound

2.1.3 Embedding

Samples were embedded using either a performed aluminium cast (Dentagen V1.5 tooth brushing machine sample cast, SyndiCAD Dental Research, Germany) or a custom-made silicone mould, shown in Figure 12.

a) b)

Figure 12 a) Image of Silicone mould and b) the aluminium cast, used to create samples

2.1.3.1 Mounting technique

At the start of the PhD Bis-acrylic composite (Protemp, 3M ESPE, Germany) was used to embed the enamel specimens into the aluminium cast. However due to the cost of the material and the large numbers of samples cold cure acrylic resin was chosen as a replacement. This material was not suited to the aluminium cast as it damaged the cast and also produced poor quality samples. This required the manufacture of a new, custom-made mould.

2.1.3.2 Moulds

There were two moulds used within the PhD. An aluminium cast produced by the manufacturer of the tooth brushing machine and a custom-made one. The aluminium cast was used initially for the first experiment with bis-acrylic composite. For the remainder of the experiments in this thesis the custom-made silicone mould was used with cold cure acrylic resin. The aluminium cast was dissembled and lubricated using a silicone mould release spray (DAS Silicone Mould Release aerosol, Electrolube). The cast was re-assembled to create a base with 3 wells for the enamel sections, which were then positioned with the buccal/lingual surface facing into the mould and the longest side of the enamel sample parallel to the shortest side of the cast. The bis-acrylic composite was then dispensed into the wells, firstly, around the enamel pieces, to stabilise them from moving when the rest of the material was dispensed. The wells were then completely filled, as quickly as possible, within the bis-acrylic composite setting time of 40 seconds. Once all the wells were filled, the lid of the cast was placed onto the body and screwed tight. Any excess composite was squeezed out from the sides and through the holes at the top of the lid. A small amount of composite was dispensed at the side and once this had set, the cast was taken apart and the samples removed. Before each casting, the cast had to be taken apart, cleaned of any composite material remnants and then re-lubricated.

The custom mould was made from silicone duplicating material (Metrosil silicone duplicating material part A and B, Metrodent Ltd UK). After testing several shapes and prototypes, a final design was decided. Six blank, unpolished, high quality moulded bis-acrylic composite samples were used as the master moulds. They were placed with the 'top' of the sample facing up, into a rectangular container (12 x 9 x 2cm) and held in place with wax. The silicone duplicating material was mixed, following the manufacturer's instructions, and then slowly poured into the container preventing air bubbles forming. Once set, the mould was carefully prised away from the container creating the base. To create a lid for the mould, the same rectangular container was used, with the master moulds removed, and it was filled with soft putty (Aquasil Soft Putty, Regular Set, Dentsply, Detrev GmbH). Once set, the block was carefully removed. To create release holes on the top of the lid, the lid was placed on top of the base and on it, the centre of the samples were marked. Using a borer of (4mm diameter), holes were placed into the lid.

No lubrication was necessary for the acrylic resin (and the bis-acrylic composite) as it did not adhere to the mould. Enamel samples were placed into the wells in the same position and orientation as for the aluminium cast. The acrylic resin was made by adding the polymer powder to the liquid monomer using a ratio of approximately 1:1 and then then poured over the samples, creating an excess and then the lid placed on top. A slight force was applied to the lid and the excess resin wiped away. A glass block with 600g of weights was placed over the top. Once set, the lid was removed and the samples removed and submersed in deionised water. The mould could be re-used immediately as the process did not leave any material on the mould. Figure 13 shows both the bis-acrylic composite and cold cure acrylic resin sample.

2.1.4 Grinding/Polishing

Samples were ground and polished (Buehler Metaserv 3000 variable speed grinder-polisher and Vector™ LC power head) with Federation of European Producers of Abrasives (FEPA) standard silicon carbide sandpaper using previously published regimes (Rodriguez & Bartlett 2010; Austin 2011). Custom-made jigs were made from bis-acrylic composite and cold cure acrylic resin to fit to the power head and hold the samples in place. A force of 10N was applied to the centre of the sample and a speed of 300rpm applied. Starting at 80-grit, for approximately 5 seconds, this produced an initially flattened area on the enamel. At this stage, the samples were individually visually inspected after drying the surface with a tissue to check that an area of enamel (approximately 1 x 2mm) had been exposed. If there was not any exposed enamel the sample was then ground again for 5 more seconds and re-checked, until there was visible exposed enamel. After which the samples were then cycled through 180 (10 seconds), 600 (25 seconds), 1200 (30 seconds), 2400 (35 seconds) and 4000 (45 seconds) grits to produce a flat, highly polished enamel surface. Samples were ground/polished in batches; with the silicon carbide disks replaced every 16 samples. When the samples were not being polished they were stored in deionised water. After the 4000 grit level the samples were placed vertically at the edge of a weighing boat with tissue paper on the base and allowed to dry naturally for at least 12 hours. This procedure removed approximately 400µm of enamel.

2.1.4.1 Measurement of amount of enamel removed

To assess how much enamel the polishing procedure removed, a digital calliper (Duratool D00325) was used to measure the thickness of the samples before and after the polishing procedure. In the development of the protocol for polishing, initially 10 samples were polished and the average amount of enamel removed was 396µm with a standard deviation of 13µm. Samples were then produced in batches of 96 and of these, 10 were randomly assessed for loss of enamel removed during the polishing procedure.

2.1.5 Reference Area

Adhesive polyvinyl tape was placed on the enamel to create a window of approximately 1mm by 3mm and two reference areas either side as shown in Figure 13. Adhesive tape was applied to a clean glass block and strips made by scoring the tape with a scalpel and a ruler. After the tape was applied the width of the worn area was measured with a ruler, if correct, the tape was pressed down more firmly, and if not, then the tape was readjusted and then rechecked.

a) b)

Figure 13 Image showing the polished samples, with adhesive tape for a) cold cure acrylic resin and b) bis-acrylic composite

2.1.6 Storage

After taping samples were stored dry in polystyrene tissue culture well plates (6 per container).

2.2 Acids

Acidic solutions were made by addition of the respective acidic solid or solution into deionised water as shown in Table 5. The pH was then adjusted with sodium hydroxide (NaOH) using a pH meter (Oakton pH 510 bench top meter). Solids were weighed using an electronic analytical scale (Mettler Toledo, XS105 Dual Range Analytical Balance) and liquids were measured using a graduated measuring cylinder.

	Concentration equivalent for all/ %		
	0.3	**0.6**	**1.0**
	Concentration/ M		
Acid	0.02	0.03	0.05
Citric Acros Organics Lots# A0303680 Product code- 110450010	3g	6g	10g
	Concentration/ M		
Acid	0.05	0.10	0.17
Phosphoric Acros Organics Lot# A0226356 Product code- 201145000	3.5mL	7.0mL	11.8mL
	Concentration/ M		
Acid	0.10	0.20	0.33
Hydrochloric Acros Organics Lot# AO275911 Product code- 124620026	8.1mL	16.2mL	27.0mL

Table 5 Table showing the amount of solid or solution added to 1000mL deionised water to make the required concentration for each solution

2.3 Erosion

The protocol for erosion cycling evolved over the course of the first year of the PhD. The main change was how the samples were immersed and removed from the acidic solution and how they were held in place. Both the original and new procedures are described below.

2.3.1 Model development

The original procedure involved individually immersing the samples into the acidic solution, agitating, and then individually removing the samples. These samples were then individually rinsed with distilled water and then added back into the solution. During the agitation, the samples had to be closely monitored as the samples moved around and sometimes moved on top of one another. The bis-acrylic composite samples did not float as they had more weight (4.5g) to keep them down. In contrast, the cold cure acrylic resin samples were lighter (3.5g) and tended to float during agitation.

As this could have led to variation in the immersion time of the samples, a new procedure was developed to ensure that all samples were immersed and removed at the same time. A bis-acrylic net was designed to hold the enamel samples which were then immersed into the acids. At the end of the immersion time the net was removed and ensured a consistent erosion time for each sample Figure 14.

a) b)

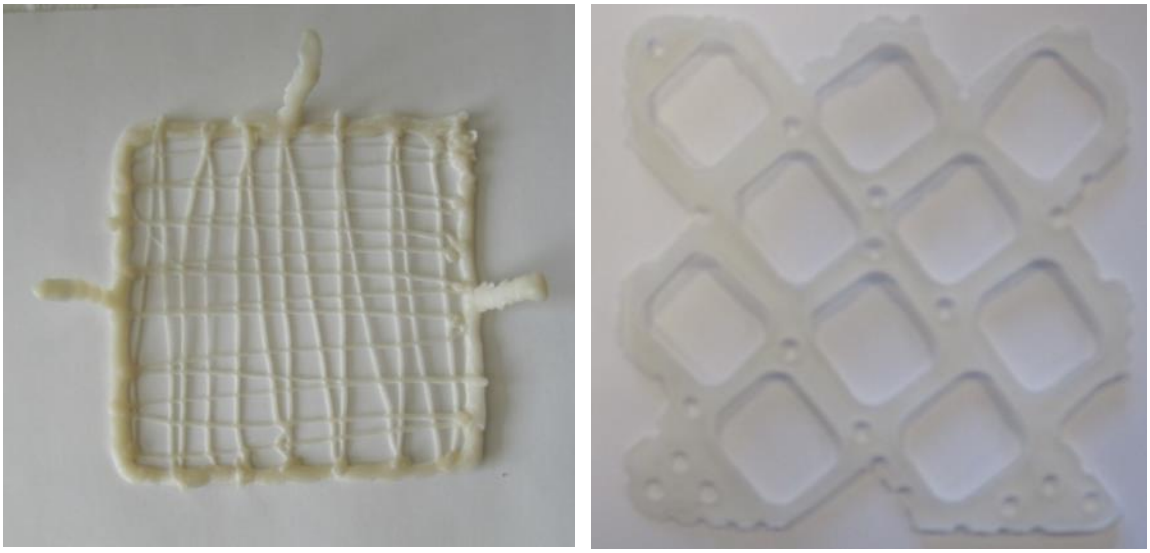

Figure 14 Picture of a) bis-acrylic composite net and b) acrylic resin jig

2.3.1.1 Drying

After the final cycle of each experimental procedure, specimens were shaken to remove any excess water and placed on tissue paper. Thereafter, specimens were allowed to air-dry for at least 12 hours at room temperate before any measurements were taken.

2.3.1.2 Cycling Procedure

Figure 15 shows the general cycling procedure for erosion-only. A single cycle consisted of immersion in the erosive solution and the time depended on the experiment. At the end of which, samples were removed and gently shaken to remove excess acid on the surface. If the cycle was repeated the samples were rinsed with deionised water using a spray bottle, shaken to remove any excess water and then either re-immersed or allowed to air dry.

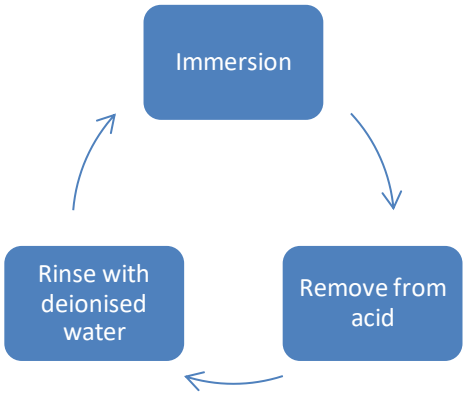

Figure 15 Generic cycling procedure for erosion only

Abrasion

2.3.2 Tooth-brushing machine

Toothbrush abrasion was performed using an automatic tooth-brushing machine (DentaGen V.1.50 Syndicad) with an Oral B P40 toothbrush (ADA reference toothbrush) at a load between 290-295g with a toothpaste slurry.

2.3.3 Toothpaste slurry

The toothpaste slurry was made to the ratio of 3 parts of artificial saliva to 1 part of non-fluoridated toothpaste (Ganss et al. 2011). The slurry was mixed in a conical flask with a magnetic stirrer and stirrer bar. The toothpaste was added slowly to the artificial saliva, allowing time to mix. Once it was fully mixed the solution was used immediately. Artificial saliva was made by addition of $CaCl_2.2H_2O$ ($7x10^{-4}$mol/L), $MgCl_2$ ($2x10^{-4}$mol/L), KH_2PO_4 ($4x10^{-3}$mol/L), HEPES (acid form) (0.02mol/L) and KCl (0.03mol/L) to deionised water and the pH was adjusted to pH 7.0 with NaOH (Eisenburger et al. 2001).

2.3.3.1 Toothpaste

The non-fluoridated toothpaste, Kingfisher Natural Toothpaste-fluoride free–Mint (Kingfisher natural toothpaste, Norwich) previously reported by our group (Rodriguez & Bartlett 2010; Austin et al. 2011) was used and contained the following ingredients: calcium carbonate,

glycerine, aqua, sodium lauryl sulphate, hydrated silica, cellulose gum, menthe piperita, citrus limonum, foeniculum and limonene.

2.3.3.2 Cycling Procedure

Figure 17 shows the cycling procedure used for erosion-abrasion experiments. One cycle consisted of immersion in the erosive solution, removal, followed by rinsing with deionised water. Six samples were individually loaded onto the tooth-brushing machine. Following a single stroke, the toothbrush was slowly moved across the surface of the enamel sample to ensure that even contact was made between the bristle and the exposed area. The 4 samples that were not being abraded were placed in a water bath. After the abrasion the samples were removed, rinsed and placed into a separate water bath. The 4 remaining samples were loaded onto the tooth brushing machine and abraded. After cycling the samples were rinsed and then placed back into the acrylic resin jig and then re-immersed. The deionised water in the water bath was replaced each cycle. Care was taken to ensure no toothpaste slurry was on the samples prior to re-immersion.

2.4 Measurement

2.4.1 Titratable acidity

Titratable acidity was calculated by measuring the volume of 0.05M NaOH solution required to raise the pH of 10mL of the acidic solution to pH 7 using a calibrated pH meter (Oakton pH 510 bench top meter). The solution was continually stirred with a magnetic stirrer (Fisher Scientific, Magnetic hotplate stirrer, USA) with the probe fully immersed in the acidic solution whilst the NaOH was added. After the addition of NaOH the solution was stirred for 2 minutes and the pH reading was noted. Initially, 5mL of NaOH was added, but as the pH approached pH 7, smaller quantities (≤ 1mL) were added. The experiment was stopped after two readings for NaOH were within 0.5mL of each other. To calculate the mmol/L the equation shown in Figure 16 was used, where C_{base} is the concentration of the base in mmol/L, V_{base} is the volume of base

required to raise the solution to the end point pH in L and V_{sample} is the volume of the sample that was titrated in L.

$$mmol/L = (C_{base} \times V_{base}) / V_{sample}$$

Figure 16 Equation for calculating titratable acidity in mmol/L

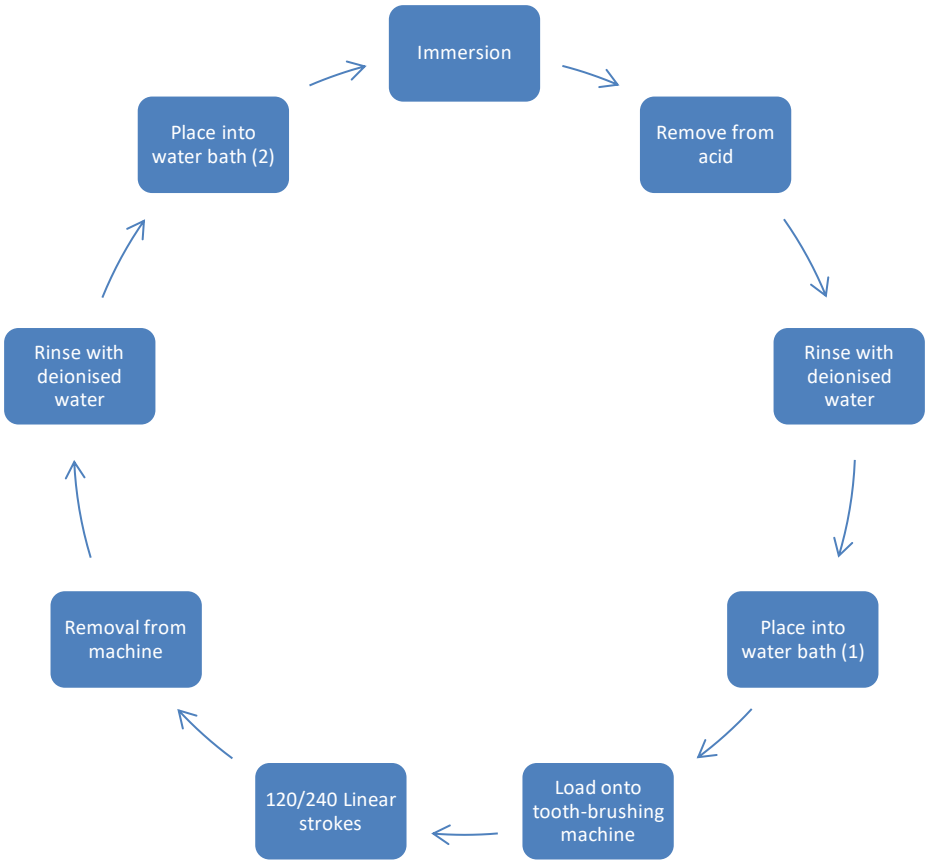

Figure 17 Cycling procedure for erosion-abrasion experiments

2.4.2 Profilometry

Profilometry measurements were obtained first, as it was non-destructive, after which the microhardness was tested. The tape covering the reference areas was carefully removed, with care being taken not to contact the enamel surface, each sample was then given a unique code. A confocal white light profilometer (Taicaan XYRIS 4000) (Figure 18) was used to measure the surface and the analysis was performed with bespoke and commercial software.

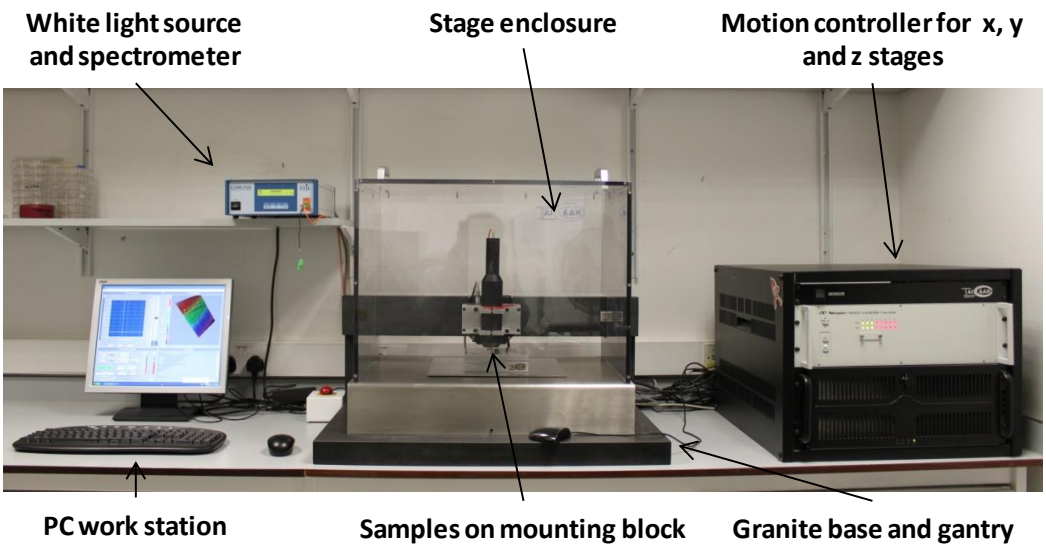

Figure 18 Image of the set up for the confocal white light profilometer

Stage	Resolution/ µm	Travel/ mm	On axis accuracy/µm	Maximum speed/ mm/s	Drivers and Bearings
x/y	0.1	100 x 100	0.4	25	DC servo Ball bearing
z	0.1	25	Not specified	n/a	DC servo Ball bearing

Table 6 Specifications for the x/y/z stages (Taicaan XYRIS 4000)

Light Source	Spot Size/ µm	Vertical resolution/ nm	Angular tolerance/ ^{o}	Gauge range/ µm	Stand-off distance/ mm	Sampling rate/ kHz
Halogen	7	10	approximately 30	350	12.7	1/4/30

Table 7 Specifications for the confocal white light sensor (Taicaan XYRIS 4000)

Table 6 shows the specifications for the Taicaan XYRIS 4000 system. The “On axis accuracy” records the deviation from absolute accuracy along a defined axis of travel. Table 7 shows the specifications for the confocal white light sensor as part of the Taicaan XYRIS 4000 system. The spot size represents the diameter of the light spot focused onto the measured surface, the

angular tolerance was the surface angle limit at which a signal could not be returned to the sensor, the gauge range was the distance at which the sensor can operate and the standoff distance was the distance between the sensor and the measured surface to bring the surface into the centre of the sensors gauge. The whole system was operated by a PC running "Windows XP" operating system and the manufactures program called Stages WL™, shown in Figure 19, was used to set up and scan the samples. The top left of the program had a manual positioning grid that corresponds to the x/y stage, and was used to manoeuvre the stage and samples to a desired location. To the left of this was a bar that could be adjusted to change the scale of the grid and perform finer movements of the stage, which was useful when trying to accurately focus on a particular point.

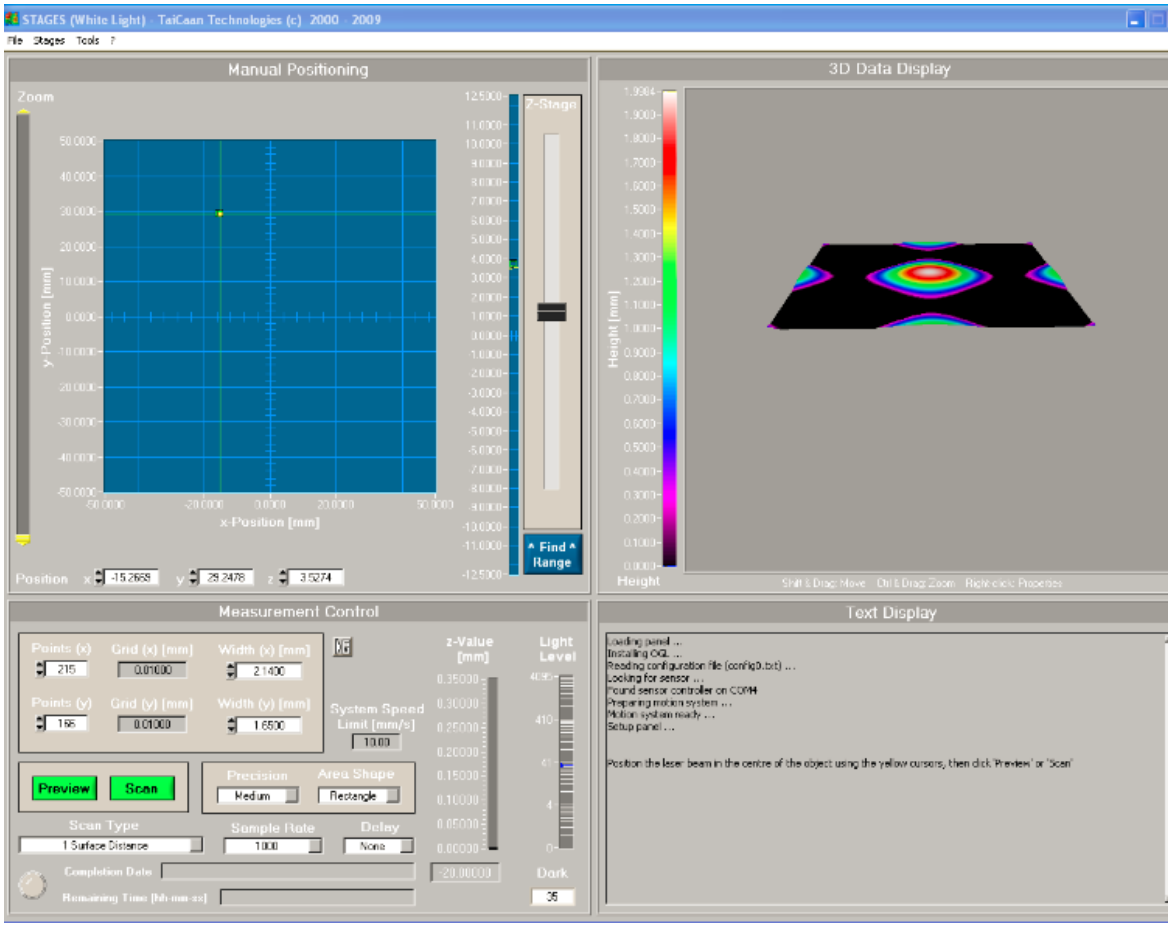

Figure 19 Screen shot of the Stages WL™ software to control the profilometer

Samples were placed onto the aluminium stage. The light source was focused onto the sample and the sensor head adjusted until the focused wavelength of light was approximately 180µm away from the surface (the middle of the sensors range). Once focused, a preview scan was taken which defined the area, at medium precision with 12 lines, producing a basic overview of the surface profile. Once the reference area had been identified the samples were scanned at full resolution, at a medium scanning speed and a 10 x 10µm x/y spacing using previously validated protocols (Austin 2011). Samples were scanned in a raster pattern, where the probe scans from left to right steadily across a sample, then moves down a set amount and repeated until the whole area had been scanned. A file consisting of a cloud of individual data points in the ASCII format was saved as a '.tai' file extension.

The step height was extracted from the raw data by one of three programs; Boddies© (Taicaan Technologies, UK) (made by the manufacturer of the profilometer), MountainsMap® (Digital surf, France) (a commercially available surface analysis software) and ImageJ (National Institute of Health, USA) (an open source image analysis software). All three programs could calculate the single line mid-point step height (SLMSH) and mean step height.

2.4.2.1 Boddies©

The 3D surface map produced from the data points collected from the scanning of the samples could contain erroneous spikes, missing values and an unlevelled surface. Firstly, the data map was cleaned by applying a function (interpolate bad data) which mathematically filled in missing data points and removed spikes. The surface was then levelled by removing the plane of best fit to produce a mathematically levelled surface. Finally, the vertical distance between the reference area and worn area was recorded as the step height loss. The protocol used in the PhD calculated a single line mid-point step height (SLMSH) to represent a measure of the loss of enamel.

2.4.2.2 MountainsMap®

The mean step height (MSH) using the ISO-5436-1 standard was calculated using MountainsMap®. Firstly the data were converted from .tai file extension to .txt extension. The surface was levelled by removing the plane of best fit using the least squares method and then converted into a series of profiles. The step height was calculated for each profile using the ISO-5436-1 method. Figure 20 shows the ISO-5436-1 formula for the surface profile calculation. The width of the worn area was calculated (W) and then split into thirds. Only the middle third of the worn area was used for the measurement (W/3). The reference area was calculated by subtracting the width of the worn area from the maximum and minimum distance away from the edge of the worn area (shown as purple lines in Figure 20). The value from the bottom of the worn area to both reference areas was calculated, and averaged to calculate the mean step height value for each profile. An average of all the step height profiles was then taken to give the mean step height (MSH).

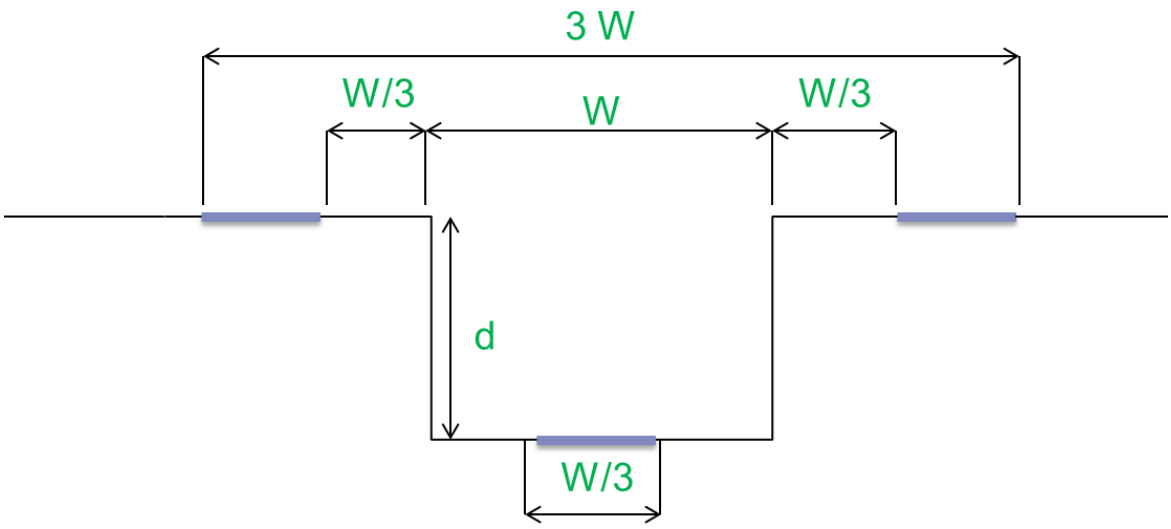

Figure 20 Schematic of the ISO-5436-1 method to calculate the step height, showing how the surface profile is broken up and the areas used for the measurement (purple lines)

2.4.2.3 ImageJ

ImageJ was used in conjunction with a macro developed by Kings College London to calculate mean step heights (Austin 2011). The macro converted the 3D data set into a 32-bit floating

point greyscale image in which each pixel represented a data point and whose grey-scale value represented the z height (the lighter grey values meant a higher z height). Therefore the dark values represented the worn area and the light values represented the reference, Figure 21. The image was levelled to a zero plane in the z-axis using the values from the lighter grey pixels. The z value (μm) for the reference and worn areas were averaged. The mean value of the reference areas was then subtracted from the worn and this difference was the mean step height for the sample.

a) b)

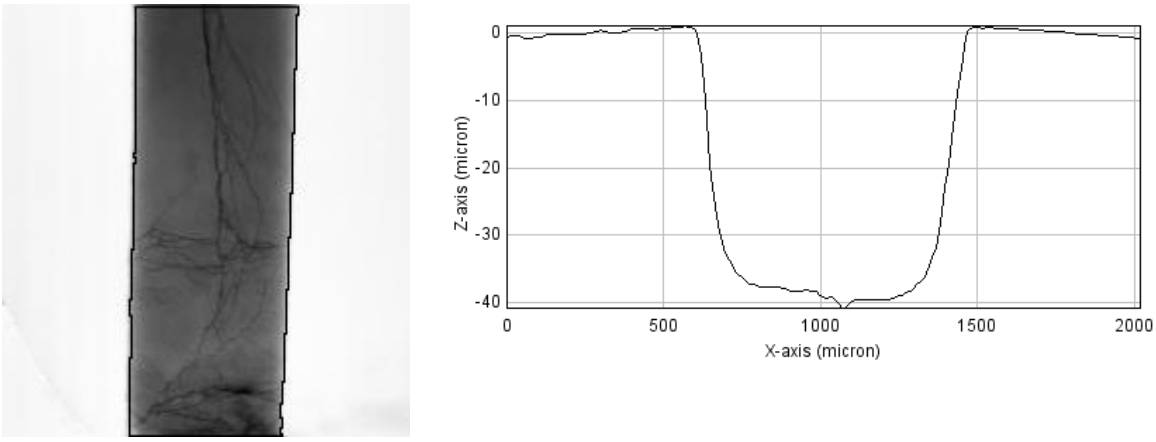

Figure 21 Schematic of the image produced by the ImageJ macro used to calculate the mean step height a) level 32-bit grey scale image (light reference area either side of the dark worn area) b) an extracted profile

Figure 22 Image of the set up for the microhardness indenter

2.4.3 Microhardness

Knoop microhardness (Struers, Duramin-1/-2) was always performed after the sample had been scanned with the profilometer, Figure 22. Samples were placed on the stage and focused onto the reference and worn areas. Indentations were made on the reference area, at least 200µm away from the worn area and in areas free of cracks. A Knoop diamond indenter, at a press load of 981.2mN and a press time of 10 seconds was used for each indentation. Each sample had 3 indentations taken 100µm apart and the values recorded on an excel spreadsheet. The sample was then moved to the worn area, so that it was in the middle, approximately equal distance from the reference areas and again 3 indentations performed and recorded.

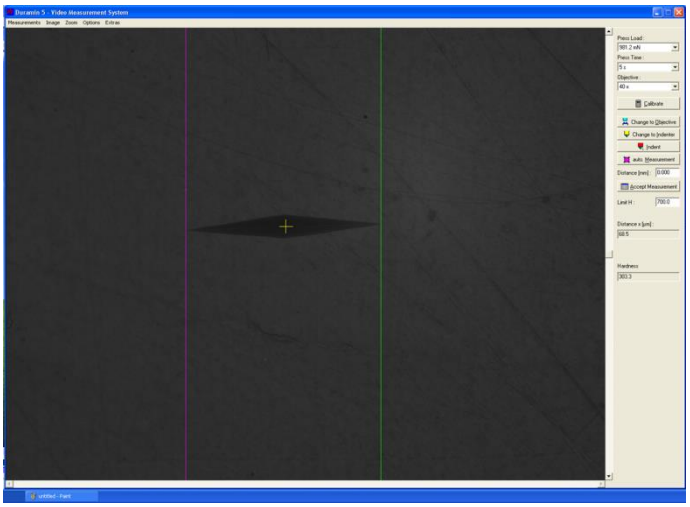

Figure 23 Screen shot of Duramin program used to measure the Knoop microhardness indentation on reference enamel, with pink and green lines at the edge of the indentation used for measurement

Chapter 3. Erosion and erosion-abrasion

3.1 Introduction

Increasing the acidic concentration of a solution and the exposure time has been shown to increase the amount of erosion (West et al. 2000; Shellis et al. 2010). The effect of abrasion on eroded enamel has been investigated and increasing the abrasion time on eroded enamel has been shown to result in increased tooth wear (Voronets & Lussi 2010) however, a linear relationship has not always been observed (Wiegand et al. 2007).

Whilst the erosive effect of citric (Barbour et al. 2003), phosphoric (Aykut-Yetkiner et al. 2013) and hydrochloric (Attin et al. 2012) acids have been studied before, the erosive effect of these acids under the same pH conditions have not been investigated. The other aim of this part of the thesis was to evaluate the software systems used to measure tooth wear and to develop protocols which were to be used later in the thesis.

3.2 Aims, Objective and Hypotheses

3.2.1 Aims

The aims were to investigate the effects of immersion times and concentrations of three common acids associated with enamel erosion, assessed by step height measurement and change in Knoop microhardness using an *in vitro* erosion and erosion-abrasion model.

3.2.2 Objectives

- To validate and compare the output of different software systems used to calculate step height change
- To investigate the effect of concentration and immersion time of citric, phosphoric and hydrochloric acid at pH 3.2 on polished human enamel and their impact combined with abrasion using 120 linear strokes
- To investigate the effect of erosion and increasing abrasion with citric acid

3.2.3 Null Hypotheses

- There is no consistency between different mapping programs when measuring the loss of enamel
- Immersion time, concentration and abrasion do not influence the amount of erosive wear

3.3 Materials and Methods

Human enamel samples were prepared using the protocols described in Chapter 2. A sample size of 10 was allocated to each group from the results of a power calculation. Table 8 shows the number of cycles, pH, immersion time and concentration of citric, phosphoric and hydrochloric acid used in each experiment. Each acid was prepared to 0.3, 0.6 and 1.0 w/v % and so the molar concentration for citric acid was 0.02, 0.03 and 0.05M respectively. For phosphoric acid was 0.05, 0.10 and 0.17M and for hydrochloric acid was 0.10, 0.20 and 0.33M. Titratable acidity was measured using a 0.05M sodium hydroxide solution to titrate 10mL of the acid.

3.3.1 Erosion-only and erosion-abrasion (EA)

Each group of 10 samples underwent 5 cycles of acid exposure for either 5, 10, 15 or 20 minutes at the concentrations listed above. Each group of samples were fully immersed in 80mL of either citric, phosphoric or hydrochloric acid and agitated with an orbital shaker (Stuart Orbital Shaker SS1) at 60rpm. For the erosion-abrasion protocol each group of 10 samples underwent, 1 cycle of erosion immediately followed by 120 linear strokes of abrasion and the process repeated 5 times. An Oral B P40 toothbrush with a force of between 290-295g was applied to each sample and abrasion created by using a tooth brushing machine (the DentaGen V.1.50 Syndicad, Germany). A toothpaste slurry, made from a non-fluoridated toothpaste and artificial saliva in a 1:3 ratio, the details of which are described in Chapter 2 was also used. For the increasing abrasion experiment samples were abraded at 30, 60 or 120

linear strokes with the same toothbrush, force and toothpaste slurry as used in the previous experiment.

	Experiment 1	*Experiment 2*
Procedure	Erosion EA 120	Erosion EA 30 EA 60 EA 120
Number of Cycles	5	5
Acid	Citric Phosphoric Hydrochloric	Citric
pH	3.2	3.2
Concentration/M		
Citric acid	0.02	0.02
	0.03	
	0.05	
Phosphoric acid	0.05	
	0.10	
	0.17	
Hydrochloric acid	0.10	
	0.20	
	0.33	
Immersion Time/ minutes	5 10 15 20	10
Measurement Techniques	Profilometry Knoop Microhardness	Profilometry Knoop Microhardness
Step Height extraction method	MountainsMap® Boddies© ImageJ	MountainsMap®

Table 8 Table summarising the factors and variables investigated of the 2 experiments in Chapter 3

3.3.2 Measurements

Profilometry and Knoop microhardness measurements were performed using the method described in Chapter 2. Following cycling, the tape was carefully removed from each sample and profilometry was performed for citric, phosphoric and hydrochloric acid immersed samples. Samples were placed on an aluminium stage, a preview scan obtained and then a full

scan was performed using the limits described in Chapter 2. The probe scanned in a raster pattern collecting data points every 10µm and step height change extracted using MountainsMap®, Boddies© and ImageJ.

Knoop microhardness change was calculated using three indentations, with a force of 981.2mN and dwell time of 10 seconds made at the centre of the worn or reference area, at least 100µm apart. The indentation length was measured and the Knoop microhardness value calculated by the program. Knoop microhardness was only performed for citric acid samples.

3.3.3 Sample Size calculation

Experiment 1

The sample size calculation for this measure was based on 3 way ANOVA for testing mean step height. Assuming an effect size of 0.3 and 80% power the study required a total sample of 197 to test the significant difference between acids, abrasions, groups and interaction at 5% level using a 2 tail test. The power calculation was carried out using gpower3.1.5. Hence it was decided to have at least 10 samples for each combination.

Experiment 2

The sample size calculation for this measure was based on 1 way ANOVA for testing mean step height. Assuming an effect size of 0.58 and 80% power the study required a total sample of 40 (10 per each group) to test the significant difference between, abrasions, at 5% level using a 2 tail test. The power calculation was carried out using gpower3.1.5.

3.4 Statistical Analysis

Intra class correlation coefficient (ICC) were calculated to compare the data from MountainsMap®, Boddies© and Image J. As the data for the erosion and increasing abrasion were normally distributed, one way analysis of variance was performed for mean step height and Knoop microhardness change followed by post hoc Scheffe tests to determine which groups were statistically significant.

Similarly the citric, phosphoric and hydrochloric acid data followed normal distribution and hence 3 way ANOVA was carried out to test the main effects of acid, abrasion and groups and the interaction effects. If the interaction was significant then further post hoc analysis was carried out using Tukey's test to find out which combinations was significant.

3.5 Results

3.5.1.1 Step height calculation

The mean calculation for step height change for 0.02M citric acid, at increasing immersion times are shown in Figure 24. For example, the mean step height (µm) standard deviation (±SD) for 5 minutes immersion for Mountains Map was 3.7 (±0.8), Boddies 3.9 (±0.8) and Image J was 3.8 (±0.6) and there were no statistical differences. Analysing the data from all concentration, immersion times and acids, the ICC comparing the three programs was 0.98 with 95% confidence interval of 0.983 to 0.986 for single measures and it was statically significant ($p<0.001$). The Cronbach's alpha value was 0.995, indicating high reliability.

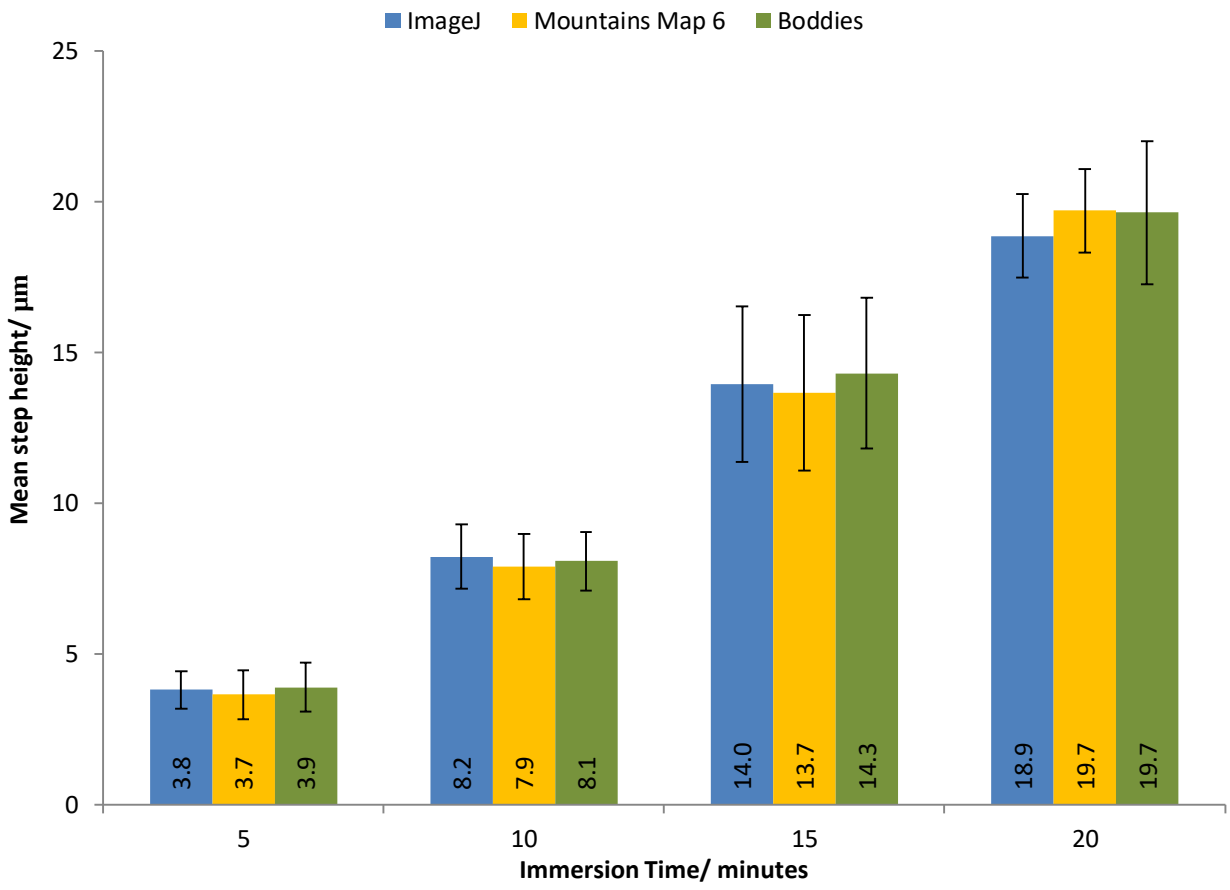

Figure 24 Chart showing the mean step height (µm) calculated by MountainsMap®, Boddies© and ImageJ, for erosion only with 0.02M citric acid with increasing immersion time

3.5.1.2 *Citric Acid*

Erosion-only-Step height

Figure 25 shows the MSH (µm) with standard deviation after erosion in 0.02, 0.03 and 0.05M citric acid for 5, 10, 15 and 20 minutes immersion time. Citric acid showed a linear increase in MSH with increasing time and concentration. For example, the lowest MSH was 3.7µm (±0.8) for 0.02M and 5 minutes immersion time and the highest was 40.9µm (±7.1) for 0.05M concentration with 20 minutes immersion time, this difference was statistically significant ($p < 0.001$).

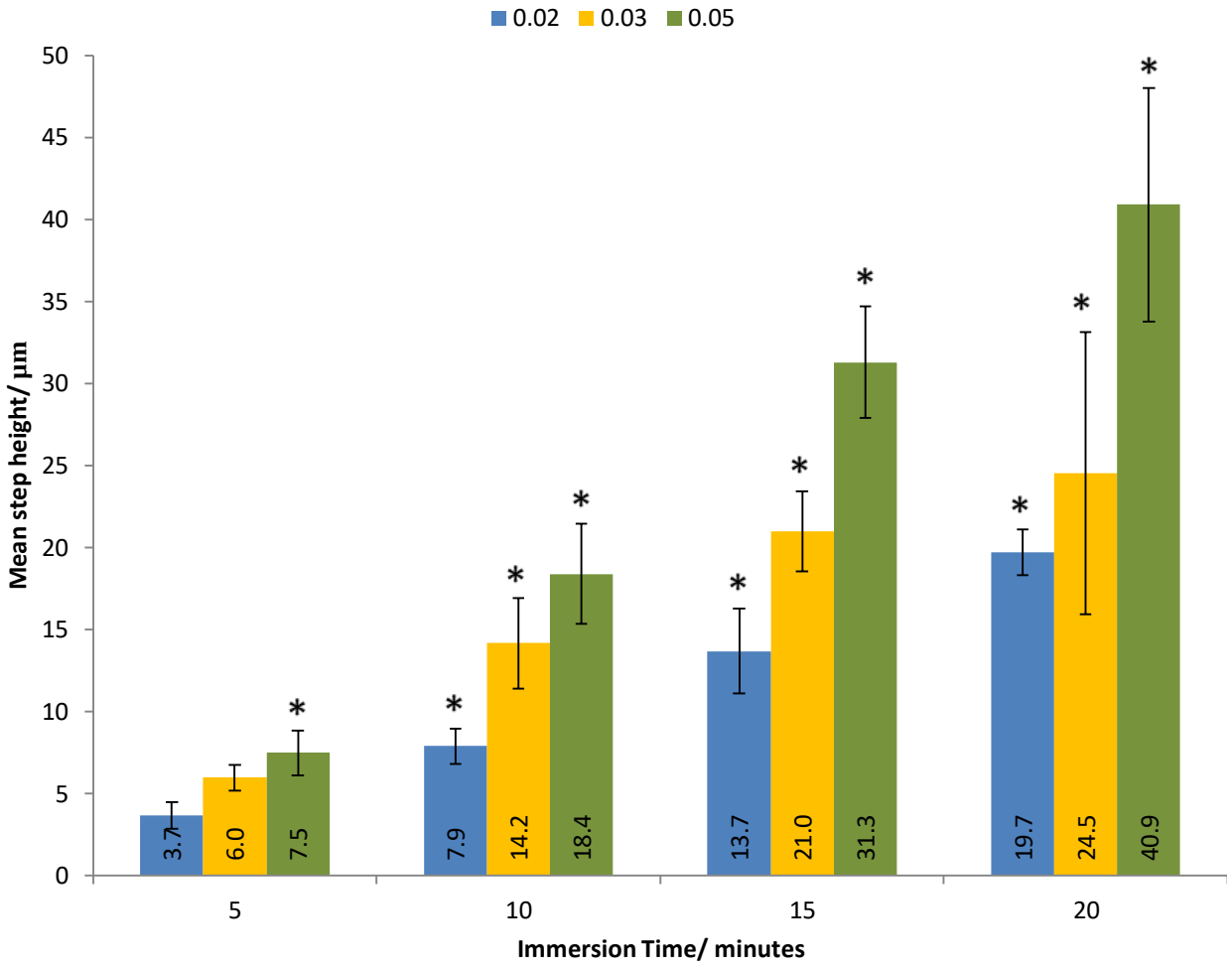

Figure 25 Chart showing the mean step height (µm) with standard deviations calculated with Mountains Map® for citric acid at various concentrations (0.02, 0.03 and 0.05M) and immersion times for erosion only as indicated. *= statistically significant ($p<0.001$) compared to 0.02M at 5 minutes immersion time

Erosion-abrasion 120 – Step height

Figure 26 shows the MSH (µm) with standard deviation after erosion-abrasion 120 in 0.02, 0.03 and 0.05M citric acid after 5, 10, 15 and 20 minutes immersion time. Increasing abrasion, under the same erosive conditions, increased the step height change. The lowest MSH was 6.7µm (±1.5) for 0.03M concentration and 5 minutes immersion time and the highest was 35.1µm (±5.0) for 0.05M and 20 minutes immersion time. At 0.02M concentration the MSH values were almost doubled compared to erosion only. On increasing immersion time with the addition of abrasion, the difference was statistically significant ($p<0.001$).

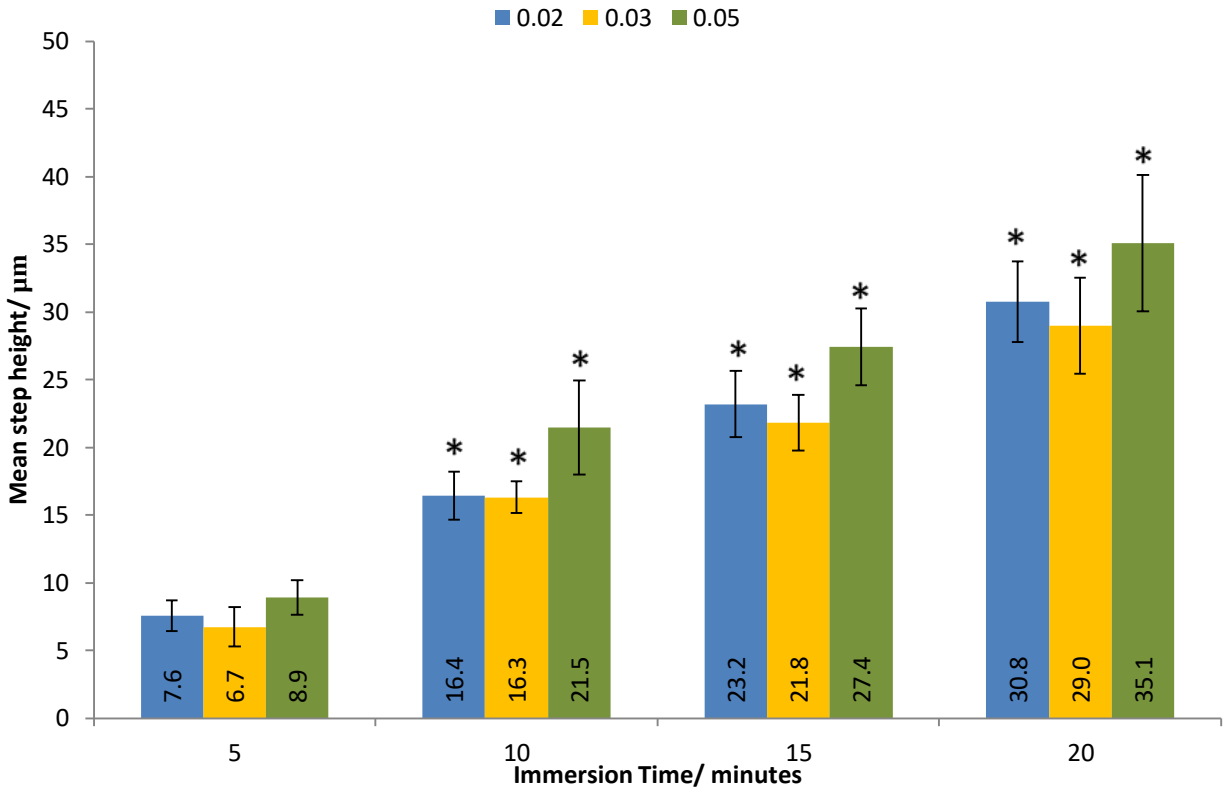

Figure 26 Chart showing the mean step height (µm) with standard deviations calculated with Mountains Map® for citric acid at various concentrations (0.02, 0.03 and 0.05M) and immersion times for erosion-abrasion 120 as indicated. *= statistically significant ($p<0.001$) compared to 0.02M at 5 minutes immersion time

Erosion-only –Knoop microhardness

Figure 27 shows the KHC (H_K) with standard deviation after erosion only in 0.02, 0.03 and 0.05M citric acid after 5, 10, 15 and 20 minutes immersion time. For erosion-only, increasing the concentration from 0.02 to 0.03 to 0.05M at 5 minutes immersion time showed an increase in the KHC (H_K) (±SD); 246.4 (±59.5) to 253.1 (±61.4) to 304.4 (±26.4) respectively and this increase was not significant (p=0.999). For 10, 15 and 20 minutes, no clear patterns were observed. Increasing the immersion time for 0.05M showed a gradual decrease in the KHC, which was not significant (p=0.980); for 0.02 and 0.03M concentration the KHC showed no patterns except for an increase in KHC for 0.02M up to 15 minutes immersion time.

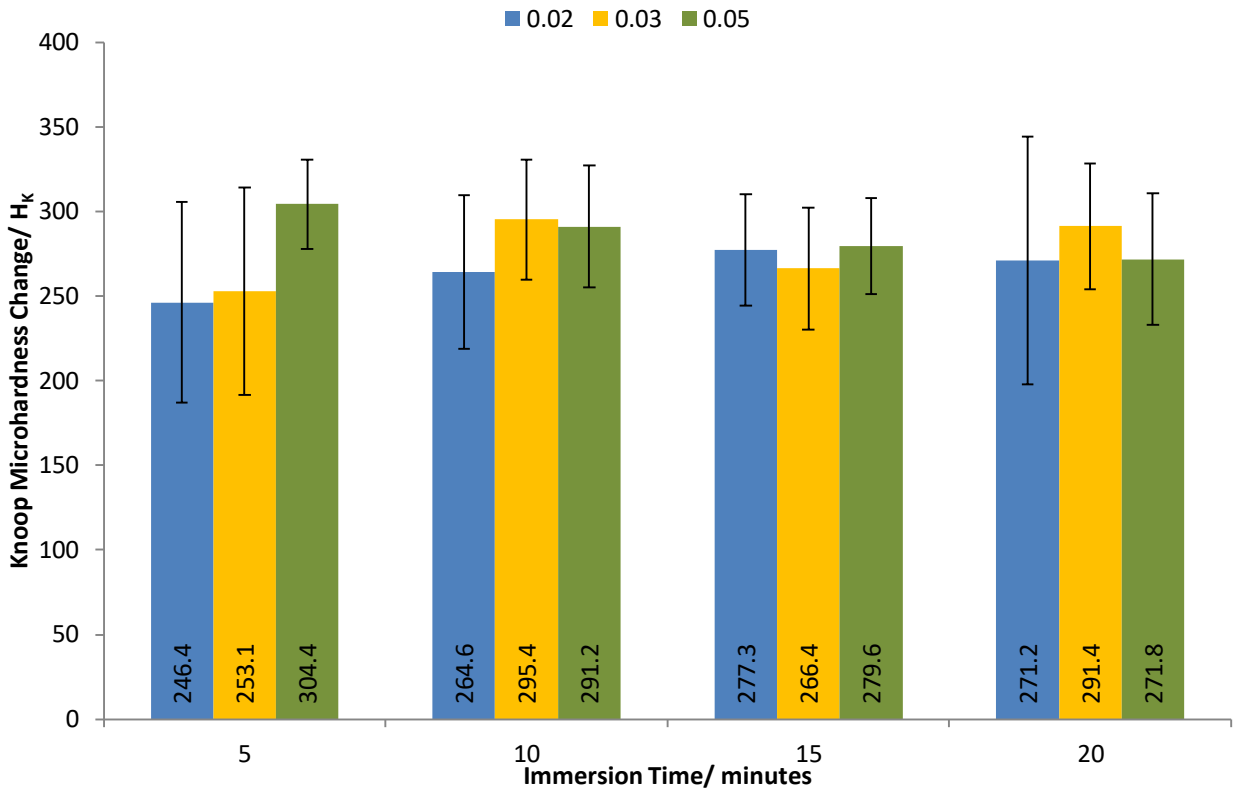

Figure 27 Chart showing the Knoop microhardness change (H_K) with standard deviations for citric acid at various concentrations (0.02, 0.03 and 0.05M) and immersion times for erosion only as indicated

Erosion-abrasion 120 –Knoop microhardness

Figure 28 shows the KHC (H_K) with standard deviation after erosion-abrasion 120 in 0.02, 0.03 and 0.05M citric acid after 5, 10, 15 and 20 minutes immersion time. For erosion-abrasion with 120 linear strokes increasing the concentration from 0.02 to 0.03 to 0.05M for 10 minutes immersion time produced an increase in KHC (H_K) (±SD); 224.4 (±39.3) to 233.4 (±40.3) to 248.5 (±45.8) respectively, this decrease was not significant (p=1.0). The highest KHC was observed for 0.05M with 15 minutes immersion time, 266.5 H_K (±25.5).

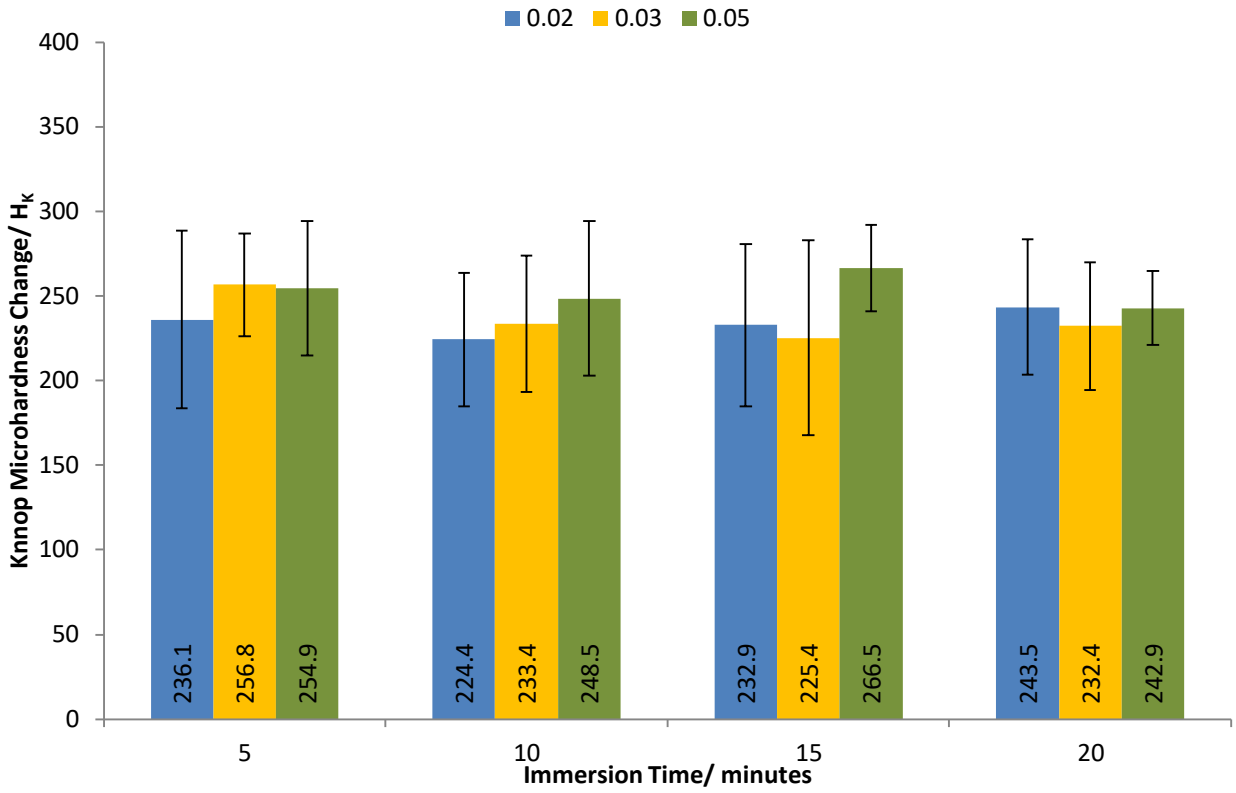

Figure 28 Chart showing the Knoop microhardness change (H_K) with standard deviations for citric acid at various concentrations (0.02, 0.03 and 0.05M) and immersion times for erosion-abrasion 120 as indicated

3.5.1.3 Phosphoric Acid

Erosion-only and erosion-abrasion 120-Step height

Figure 29 a and b show the MSH (µm) with standard deviation after erosion-only and erosion-abrasion 120 in 0.05, 0.10 and 0.17M phosphoric acid after 5, 10, 15 and 20 minutes immersion time. For all erosion and erosion-abrasion groups increasing the immersion time and concentration increased the MSH and the differences were statistically significant ($p< 0.05$). The highest MSH was 43.2µm (±2.6) for erosion-abrasion with 120 linear strokes at 0.10M concentration and 20 minutes immersion time; the lowest was 1.7µm (±0.6) for erosion only at 0.05M concentration and 5 minutes immersion time.

Compared to erosion-only, the addition of 120 linear strokes of abrasion produced an increase in the MSH. For example, increasing the concentration at 20 minutes immersion time the MSH increased from (mean step height (µm) (±SD)); 9.7 (±0.7), 24.9 (±3.0) and 39.2 (±4.3) respectively. Compared to citric acid, (0.02M for citric acid and 0.05M for phosphoric acid) phosphoric acid produced lower MSH values. The addition of abrasion, produced statistically increased step height change ($p<0.05$) for most concentrations.

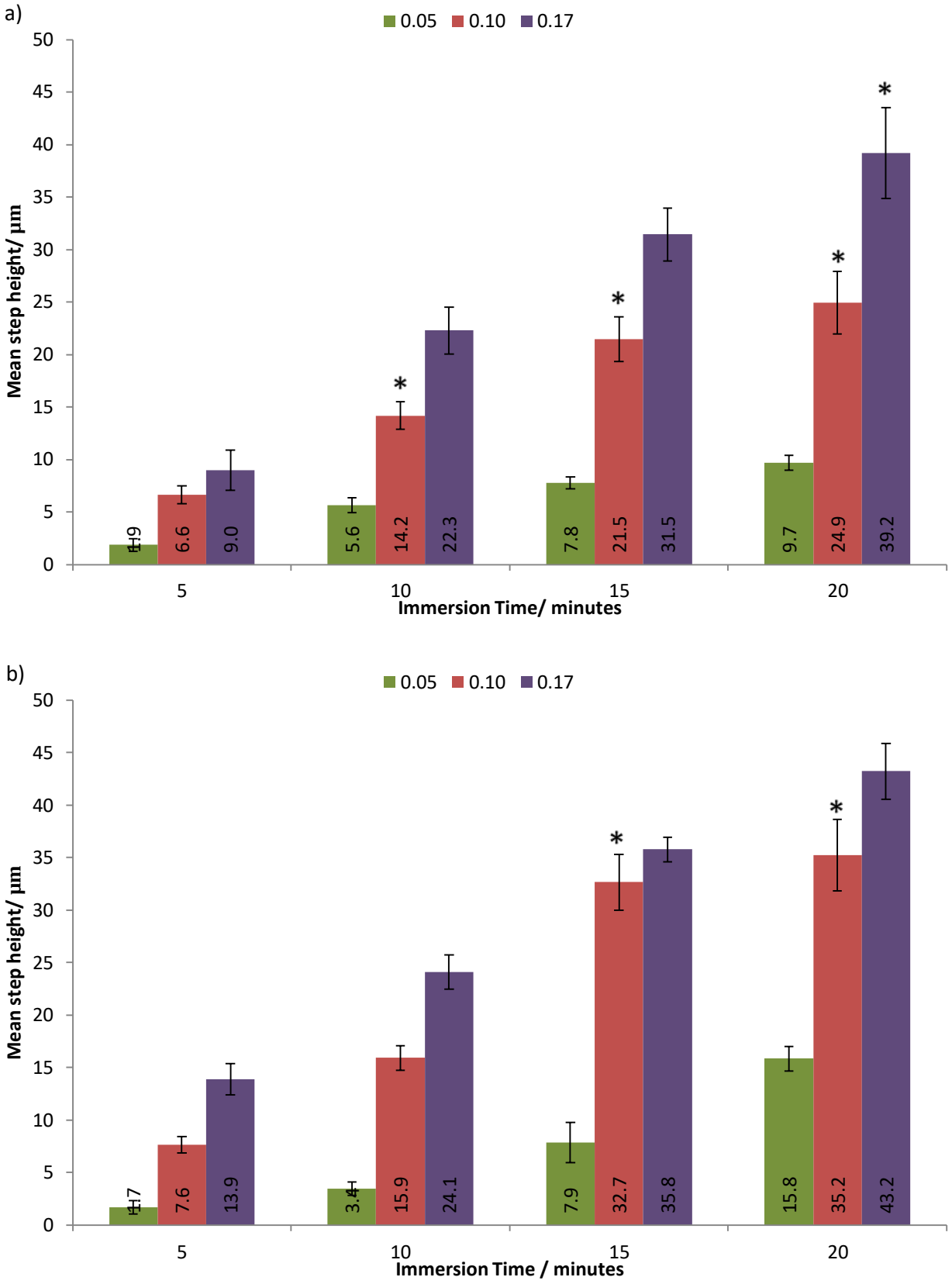

Figure 29 Chart showing the mean step height (µm) with standard deviations calculated with Mountains Map® for phosphoric acid at various concentrations (0.05, 0.10 and 0.17M) and immersion times for a) erosion-only and b) erosion-abrasion 120 as indicated. *= statistically significant ($p<0.001$) compared to 0.05M at 5 minutes immersion time

3.5.1.4 Hydrochloric acid

Erosion-only and erosion-abrasion 120-Step height

Figure 30 a and b shows the MSH (µm) with standard deviation after erosion-only and erosion-abrasion 120 in 0.10, 0.20 and 0.33M hydrochloric acid after 5, 10, 15 and 20 minutes immersion time. For erosion-only, increasing the immersion time for each concentration increased the MSH. But within each concentration the step height change for 0.20M was generally higher for each time period.

For erosion-abrasion with 120 linear strokes, at all concentrations, increasing the immersion time increased the MSH. Similar to the data from erosion-only the 0.20M concentration produced the highest step height change. Compared to erosion-only there was a slight decrease in step height change with abrasion but the difference was not statistically significant ($p > 0.05$).

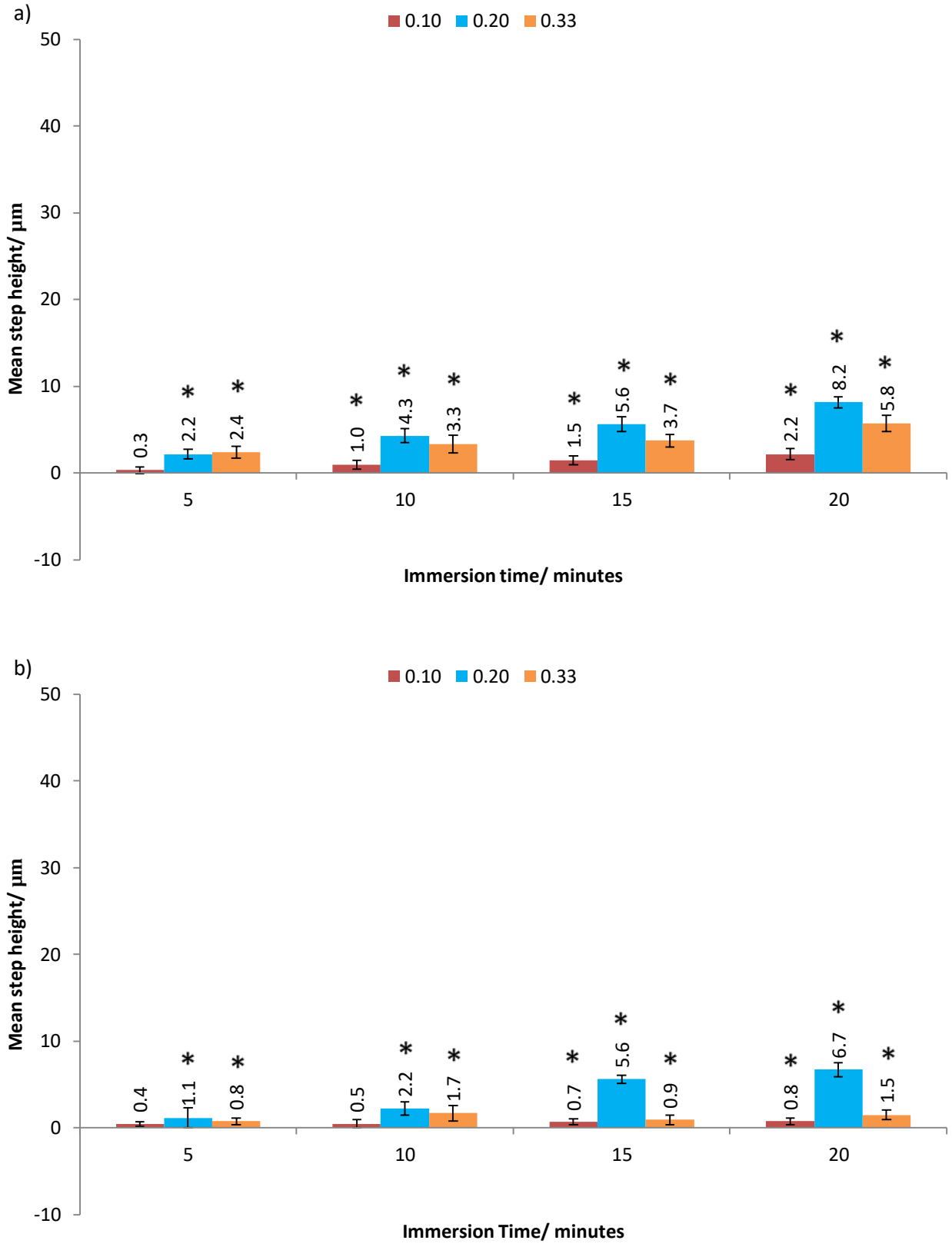

Figure 30 Chart showing the mean step height (µm) with standard deviations calculated with MountainsMap® for hydrochoric acid at various concentrations (0.10, 0.20 and 0.33M) and immersion times for a) erosion and b) erosion-abrasion 120 as indicated. *= statistically significant ($p<0.001$) compared to 0.10M at 5 minutes immersion time

3.5.2 Increasing abrasion

The citric acid erosion (0.02M and 10 minute exposure) protocol remained the same as previously described in section 3.3.1 but samples were subjected to an increasing number of linear strokes of 30, 60 or 120 strokes.

Figure 31a and b shows the mean step height and Knoop microhardness change results with standard deviations for the increasing abrasion experiment. For erosion-only, a MSH of 8.1µm (±1.0) was produced. There was no statistical difference between 30 and 60 strokes (7.6µm (±0.9) and 8.6µm (±1.1)). However, with 120 linear strokes the MSH was doubled compared to erosion-only, to 16.5µm (±1.9,) and this difference was statistically significant ($p<0.001$).

Knoop microhardness

For KHC, erosion-only produced a value of 264.4H_K (±33.6). Unlike the data from the step height change there was a significant change from erosion only to 30 and 60 strokes ($p<0.001$) but not for 120 strokes.

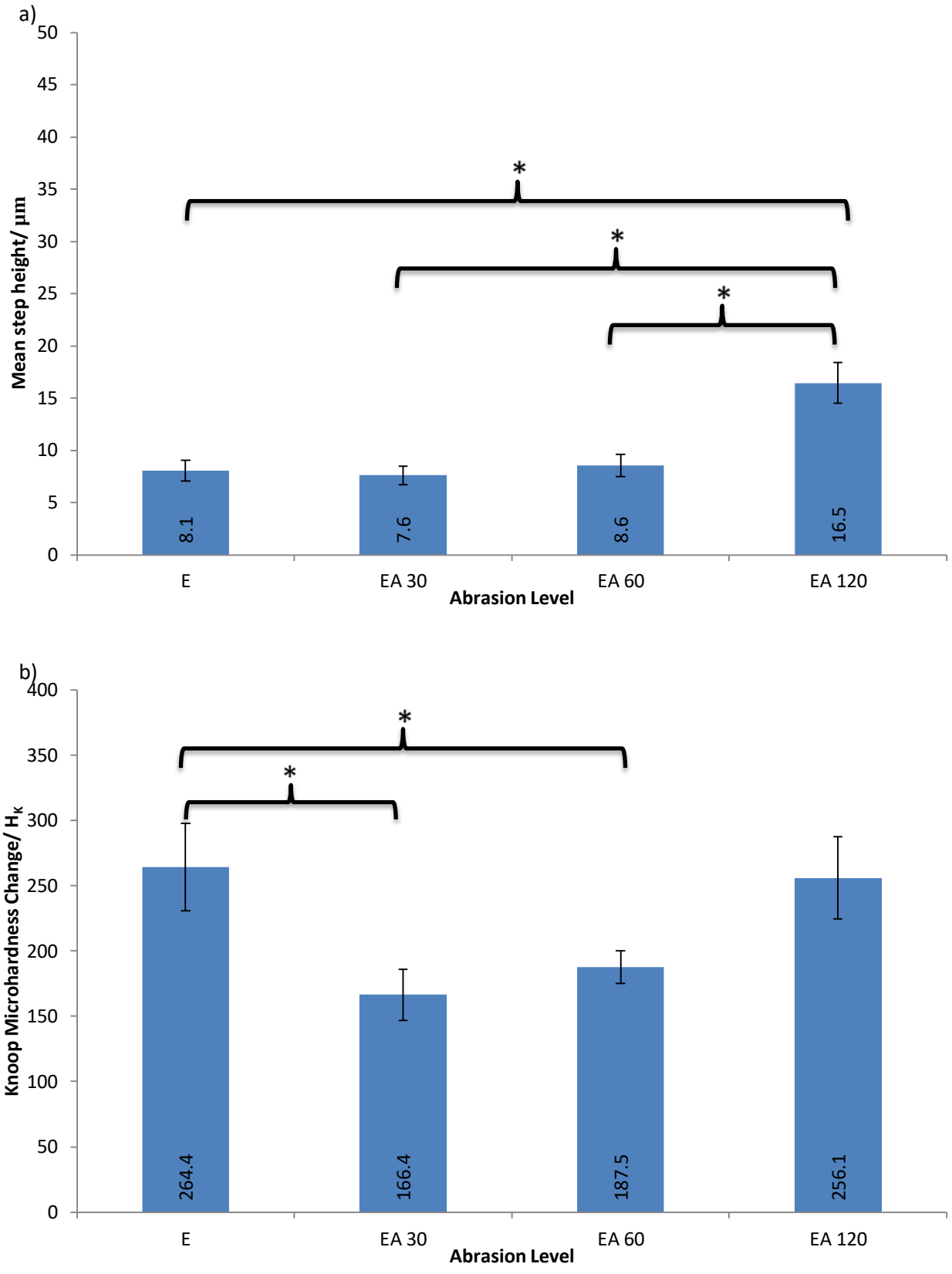

Figure 31 a) Mean step height (µm) and b) Knoop microhardness change (H_K)with standard deviations for citric acid (pH 3.2) at 0.02M concentration and 10 minutes immersion times for erosion, erosion-abrasion 30, 60 and 120. * = statistically significant ($p<0.001$)

3.6 Discussion

Overall the effect of type of acid, immersion time, concentration and the addition of abrasion statistically increased the mean step height but the effect on microhardness was not statistically significant. This difference between the outcomes probably reflects the acidic nature of the experimental model, which was designed for profilometry. Current concepts suggest that microhardness is a better value for early erosive changes whilst profilometry better represents more severe conditions or a combined effect of erosion and abrasion. For this reason microhardness data was only collected for the citric acid model.

The initial investigations sought to establish if three commonly used analysis programmes produced different data. Each program analysed the step height change differently. Boddies© calculated the SLMSH, as a single step height taken manually across an arbitrarily determined mid-point of the sample. For ImageJ and MountainsMap® the mean step height was calculated automatically along the wear scar, but worked in slightly different ways. ImageJ used a custom-made macro, which automatically selected the reference and worn areas by converting the data points into a 2D greyscale image, where the grey value of each pixel represents the z value (height) of that data point. The surface was levelled and step height automatically calculated from the reference and worn areas. MountainsMap® calculated the step height based of the ISO standard 5436-1. Despite the differences in the methods used to calculate the step height they all produced consistent measures that were statistically not different ($p<0.001$). This high level of agreement meant that any three of the methods could be used for step height calculation.

For the rest of the thesis, MountainsMap® was used to calculate the mean step height and for statistical analysis. This software was chosen as MountainsMap® has the ISO standard 5436-1 and gave a convenient standardisation for the measurements. The SLMSH is prone to human bias as the operator has to physically choose the reference and measure the worn areas.

However the high level of agreement of this technique compared to the others showed that this was still a valid method, but Mountains Map was selected as the most appropriate technique.

Polishing enamel using the protocol produced a sample with a flatness tolerance of ±0.6µm. Therefore, low values of step height loss of <1.2µm were difficult to discriminate compared to the reference areas. At these values, measurements taken were at the extreme resolution of the instrument with the current polishing procedure. For this reason, the model used in the protocol was selected to be quite erosive. The limitation of the measurement system would influence the model design. Higher resolution profilometers are available but increasing resolution introduced practicality issues and time constraints on the area to be scanned. The same size of area scanned on a higher resolution profilometer would take longer and require a larger file size. For profilometry, a flatter surface would aid the measurement and allow for smaller vertical distances to be more accurately measured.

The calculation of a single line and mean step heights produced similar values, so it was assumed that the wear across the exposed surface was uniform. If it was not uniform, then a larger discrepancy between the data from a single line step height and the mean step height might be expected. Experiment 1 showed that at the same pH (3.2) citric and phosphoric acid produced a greater MSH and therefore erosion, than hydrochloric acid. For both citric and phosphoric acid, increasing the concentration and immersion time increased the MSH, which was expected. However, for hydrochloric acid, this result was not always observed. For hydrochloric acid, increasing the immersion time increased the MSH for all concentrations. However, increasing the concentration did not always increase the MSH. With the addition of abrasion, an increase in the MSH was expected however this did not always happen and in some cases a decrease in the MSH was observed. Citric acid showed an increase in MSH for

0.02 and 0.03M concentration whereas phosphoric acid showed an increase for 0.05 and 0.17M. Hydrochloric acid did not show an increase in MSH with addition of abrasion.

Whilst there have been papers comparing different acids; hydrochloric vs. citric (Attin et al. 2012), citric vs. phosphoric (West et al. 2000; Aykut-Yetkiner et al. 2013) and citric, hydrochloric and phosphoric (different concentration for citric vs. phosphoric) (West et al. 2001); none have compared them in the same study under the same pH and cycling conditions. In this study citric, phosphoric and hydrochloric acid were chosen as these are the main acids identified with erosion (Lussi et al. 2012). The concentrations of 0.02 and 0.05M were chosen as citric acid has a concentration of approximately 0.02M and pH of approximately 3.2 in ready-to-drink juices, whereas a concentration of approximately 0.05M is found in orange juice (West et al. 2001; Shellis et al. 2005). A middle concentration of 0.03M was used to observe a gradual increase. Naturally acids, such as phosphoric and hydrochloric, do not occur at these concentrations and at pH 3.2. However in this study, these acids were also adjusted to pH 3.2 to allow a comparison under the same conditions. The immersion times were chosen based on pilot work, which gave a measurable amount of step height loss but also compared well with the range of clinically relevant times (20-120 minutes) (Schlueter et al. 2005). Buffering the acid to pH 3.2 allowed control of the acidic solutions but in the case of hydrochloric acid it was buffered so much that low values for erosion were obtained. Conversely, if a more realistic value for the concentration of hydrochloric acid had been chosen it would not have been possible to directly compare the different acids.

For the tooth brushing machine, one stroke takes 1 second and therefore the 120 linear strokes equates to 2 minutes of brushing for a single tooth. The total brushing time in our study equated to 600 seconds, which if assuming that each tooth is brushed on average for 10 seconds, twice a day (Heintze et al. 2010) then the 120 linear strokes represented a month (30 days) of brushing. Therefore, 30 linear strokes represent approximately a week (7.5 days) of

brushing and 60 linear strokes represents approximately 2 weeks (15 days) of brushing. Ganss et al. investigated tooth brushing habits of adults and found that the mean brushing force was 234g (± 71) (Ganss et al. 2009). However this range varied from 150g-720g. Our value of 290-295g was chosen as it was in the middle of the range. The weight was also chosen based on what could be practically achieved with the tooth brushing machine. A weight higher than 300g would bend the tooth brush and thus affect how the tooth brush head would contact the enamel surface.

A toothpaste slurry was used to make the abrasion more clinically relevant. Clinically, brushing teeth is performed in combination with toothpaste and saliva. Non-fluoridated toothpaste was selected to remove any protective effect from fluoride. Natural saliva would have been more clinically relevant but artificial saliva was chosen as it allowed greater standardisation across all of the experiments. Artificial saliva has been used in previously published studies and it is unlikely that its inclusion in the toothpaste slurry would have had a significant remineralising effect on the eroded enamel. Eisenburger et al. reported 0.7µm (± 1.0µm) gain to the surface after a remineralisation time of 60 minutes in artificial saliva (same composition as the one used in this study) (Eisenburger et al. 2001). In our study, samples were exposed to the artificial saliva for a maximum of 20 minutes and so any increase, if present, was not detectable by the profilometer.

Non-contact profilometry was selected as the primary measurement instrument as it is used extensively in erosion studies and is seen as a reliable tool in dental research (Paepegaey et al. 2013; Attin & Wegehaupt 2014). Knoop microhardness was chosen as a secondary measurement instrument to assess the change in hardness. It has been used in previous studies and is shown to be a useful way to study early enamel erosion and surface softening (Lussi & Jaeggi 2006; Shellis et al. 2011). The Knoop diamond was chosen as it penetrates the surface less than a Vickers diamond and is therefore more sensitive to early enamel erosion.

Less penetration of the surface also meant that the underlying harder enamel would have less of an influence on the measurement. The press time and force were chosen based on pilot work that resulted in a measureable indent that was not too small or too large on the reference enamel. The same settings were used for the worn enamel so that a comparison and then a change could be calculated from the reference enamel. The machine used had load cell technology. This is more accurate than mechanical weights, as it is free from influences of friction and inertia within the system.

The effect of time, concentration and the different acids was largely predictable. Generally, increases in time and concentration for each acid increased the mean step height. The pattern was clearer for citric and phosphoric acids but slightly different for hydrochloric acid, which at 0.20M had a greater step height change than either 0.10 or 0.33M. This difference is not easy to explain as the values of the step height were within the resolution of the system and polishing procedure. It appears that at 0.20M the polished enamel behaved differently and was more susceptible to erosion.

The addition of abrasion had varying results. For citric acid the addition of 120 strokes increased the mean step height and in some situations doubled the value. The impact of 0.02M citric acid was more profound compared to erosion only. Like citric, phosphoric acid, showed a generalised increase in mean step height with abrasion but the results were not as clear. Again the effect of the change in concentration and time will be due to the overall availability of the hydrogen ions to erode the hydroxyapatite. The impact of hydrochloric acid was less clear as a result of the low level of hydrogen ions available after the high amount of buffering needed.

Another possible reason for the differences with abrasion was that the surface layer of enamel was softened, penetrating a few micrometres. The depth would be dependent on the type and concentration of the acid (Hemingway et al. 2006). The tooth brush abrasion would remove

this softened outer layer exposing the underlying harder, sound enamel which is more resistant to the abrasive forces (Addy & Hunter 2003; Hunter et al. 2002; Eisenburger & Addy 2002). Another possibility is that the erosive process alone, was enough to soften and then remove the demineralised enamel, so during abrasion, the tooth brushes were contacting hard enamel, which would have minimal effect. In the case for 0.02M citric acid; the MSH almost doubled from erosion to erosion-abrasion with 120 linear strokes. Wiegand et al. also observed that abrasion produced smaller and non-significant differences compared to erosion-only controls. Whilst direct comparisons cannot be made to their study they used a medium filament, manual tooth brush, with 2.5N force (we used between 2.8-2.9N) applied with a total of 4000 linear strokes (we used a total of 1200), citric acid (pH 2.3), 25 minutes total immersion time and they included a remineralisation stage and contact profilometry. Even with the lower pH, higher linear strokes and contact profilometry (which can give larger step height values) they still found the addition of abrasion to cause little change (Wiegand et al. 2006).

Ganss et al. designed part of an erosion-abrasion study to produce a MSH of 10-15µm. They achieved this with 0.5% citric acid (pH 2.5), 120 minutes of demineralisation with agitation and 300 seconds of abrasion at a force of 200g with an ADA reference toothbrush. Although direct comparisons cannot be made to our study, our study achieved this MSH with 50 minutes demineralisation, 600 seconds of abrasion and 290g of force. Our study had longer abrasion and force so one would expect a higher MSH however our study used citric acid at pH3.2 which has been shown to be much less erosive than pH 2.5 (Ganss et al. 2012; Barbour et al. 2003).

The Knoop microhardness showed varied results with large standard deviations. Following this it was decided that this analysis would not be performed on either phosphoric or hydrochloric acids. Microhardness is designed for smooth, polished and highly reflective metal surfaces. Polished enamel fulfils some of these requirements but the variability in the structure of

enamel means variation in the data. On worn or eroded enamel, the outline of the indent was often rough and unreflective reducing the reliability of the measurement under the conditions used in these models. For early erosion and loss less than 14μm microhardness appears to more accurate as the edges of the indentation are more clearly defined and has been described by previous authors (Collys et al. 1992).

Our results are similar to previous work by other researchers for both citric and phosphoric acid. However subtle differences were observed and can be explained due to the variation in the protocol. Eisenburger et al. used 0.3% citric acid at pH 3.2, with gentle stirring and an erosion time of 2 hours on human enamel which produced a mean erosion depth of 20.4μm (±SD 3.5μm) (Eisenburger et al. 2001) whereas our model produced 18.87μm depth at the same concentration but with only 20 minutes immersion. The difference could be explained by the agitation method however it is difficult to accurately say this was the reason. Increasing the concentration of citric acid increased the MSH in this study. Shellis et al used the dissolution rate of enamel, measured with a pH stat and also observed that increasing the concentration of pH 3.2 citric acid from 0.3 to 1.0% increased the amount of dissolution and therefore the amount of erosion (Shellis et al. 2010).

At the concentrations and immersion times investigated in this study, citric acid was observed to be more erosive than phosphoric acid. Muller et al. also observed this in 1949 and concluded that phosphoric acid at pH 3.25 was less erosive on enamel than citric acid (Muller & Gortner 1949). Several studies have compared citric to phosphoric acid using calcium/phosphate release (Hannig et al. 2005) or profilometry (West et al. 2000; Aykut-Yetkiner et al. 2013). At the same pH and concentrations within the same studies both Aykut-Yetkiner et al. and West et al. observed that phosphoric acid produced higher MSH compared to citric acid, this generally supports our results at the 10 and 15 minutes immersion times. A study by Beyer et al. used both citric and phosphoric acid (amongst others) in their experiment

to assess the difference in erosion between the acids but used 'equivalent sensorial acidic taste' to adjust the acids, which resulted in different concentrations and pH between the acids and so no comparison could be made (Beyer et al. 2011).

The data for step heights from the hydrochloric acid models were close to or at the maximum resolution of the profilometer with the polishing protocol. Due to the minimal step heights recorded for hydrochloric acid, the profilometry data, when less than 1μm, should be treated with caution. Although the mathematical accuracy of these instruments is often quoted below 1μm these are generally tested on metals, which produce a much flatter surface. Generally, there was an increase in step height loss with time and concentration but the effect of the strength of the acid (being weakened due to the buffering) overwhelmed these factors.

The low step height data for hydrochloric acid reflects that at pH 3.2 the potential to cause erosion under these conditions is low. West et al. showed similar results and notably at just below pH 2 hydrochloric acid starts to induce much higher levels of erosion as the influence of the sodium hydroxide buffer becomes less significant increasing the availability of hydrogen ions (West et al. 2001). The native pH of hydrochloric acid is approximately 1.2-2 and in this study it was 3.2. The titratable acidities at this pH are very low and combined with its inability to chelate to the calcium could be the reason for the low step height loss. At pH 3.2, hydrochloric acid is so weakened it become less erosive as the supply of alkaline sodium hydroxide needed reduced the amount of hydrogen ions dramatically.

The results from increasing the level of abrasion (in terms of linear strokes) to 60 linear strokes did not produce a significant increase in the MSH compared to erosion-only, however there was a significant difference at 120 strokes. Both 30 and 60 linear strokes showed almost no effect on the MSH compared to erosion only. The low levels of abrasion produced almost no increased step height loss compared to erosion-only. The total amount of linear strokes that the enamel samples were subjected to after the 5 cycles were: 150 (corresponds to 30 per

cycle) and 300 (corresponds to 60 per cycle). This shows us that in our model, somewhere between 300 and 600 linear strokes are when the abrasion began to produce greater levels of surface loss. Below 60 linear strokes it appears that there is insufficient time to remove a layer of softened enamel. It is difficult to find other studies that have investigated low levels of *in vitro* erosion-abrasion similar to our study. Other studies investigating *in vitro* erosion-abrasion have done so with; fluoridated toothpastes with varying relative dentine abrasivity, different forces, different toothbrush styles and inclusion of remineralisation steps. A study by Wiegand et al. studied the effects of 10, 20, 50, 100 and 500 brushing strokes but they performed brushing before the erosive challenge (Wiegand et al. 2014).

3.7 Summary

Erosion-only experiments showed an increase in mean step height with increasing concentration and immersion times and differing results with the three acids. The pH-adjusted acids gave greater control over the experiment. However, in the case of hydrochloric acid, the acid required such a high adjustment, that it lost the capacity to erode enamel. Erosion followed by abrasion is a complex process. The machinery to simulate tooth brush abrasion is a very simplified representation of the clinical situation. The results for the most part reject the null hypotheses.

Non-contact profilometry can be used to accurately measure step height loss. MountainsMap® with its built in ISO 5436-1 standard makes this the preferred choice. Knoop microhardness measurements maybe informative at low step height values but at high step height loss values the data becomes questionable and almost unusable.

Chapter 4. Model Variables

4.1 Introduction

There is variability in the description of the methods used by different researchers. On further analysis not only did the procedure differ between investigations but also the detail of the chosen methods. Some authors accurately described each stage whilst others left out information. The three main areas that show variability were the sample preparation, erosive cycling protocols and the measurement technique. Three variables were investigated which were associated with the sample preparation and four variables associated with the erosive cycling. This allowed for a direct comparison of what the effects of changing the different variables had on the profilometry and Knoop microhardness.

4.2 Aims, Objectives and Hypothesis

4.2.1 Aims

The aims were to investigate the effects of different model variables on *in vitro* erosion using profilometry and Knoop microhardness.

4.2.2 Objectives

To assess the effects of

- Tooth type (molar/premolar) and tooth surface (buccal/lingual)
- Ultrasonication
- Storage of samples
- Mode and speed of agitation
- Rinsing between cycles
- Volume of acid
- Position of sample in the solution

4.2.3 Null Hypotheses

- Tooth surface (Buccal/ lingual) or type (molar/ premolar) does not affect the step height or microhardness change
- Ultrasonicating the samples after polishing does not affect the step height or microhardness change
- Storing samples; dry, in deionised water for 1 hour or 24 hours prior to erosion does not affect the step height or microhardness change
- Using Orbital, Gyro and See-saw agitation at 30, 40, 60 and 70rpm does not affect the step height or microhardness change
- Rinsing the samples with a spray bottle or a container between erosive cycles does not affect the step height or microhardness change
- Increasing the volume of acid from 80 to 100mL does not affect the step height or microhardness change
- Facing the enamel surface 'up' or 'down' in the solution does not affect the step height or microhardness change

4.3 Materials and Methods

4.3.1 Erosion

All experimental groups in this section contained 10 enamel samples made from a random mixture of molar, premolar, buccal and lingual surfaces using previously described protocols (section 2.1). Each group was eroded with 80mL of 0.02M citric acid adjusted to pH 3.2. The citric acid was made as described in Chapter 2 and samples underwent 5 cycles of erosion also described in Chapter 2. The samples were immersed for 10 minutes for each cycle, agitated at 60rpm with an orbital shaker and rinsed with a water spray bottle. The agitation and speed, rinsing and volume were varied for the 'agitation and speed', 'rinsing' and 'volume' experiments, the details of which are described below.

4.3.2 Tooth Surface/Type

The enamel sections were taken from buccal and lingual surfaces of molar and premolar teeth, using previously described protocol (Chapter 2, section 2.1), were subjected to erosion cycling.

4.3.3 Agitation and speed

Erosion was performed using three standard stirrers (Gyro, Orbital and See-saw) using the cycling described above and compared to a control group. Each stirrer was investigated at four speeds (30, 40, 60 and 70rpm). The three commercially available stirrers had a different mode of action, a Gyro (Stuart 3D gyratory rocker SSL3; 3D up and down, circular motion from a central point), Orbital (Stuart Orbital Shaker SS1; 2D circular orbital motion) and See-saw (Stuart See-saw rocker SSL4, 3D; up and down rocking action from a central pivot). The control group was an unstirred solution of citric acid on a flat bench.

4.3.4 Rinsing

The influence of rinsing after cycling was investigated using four methods (spray rinsing 30 seconds, container rinsing 30 seconds, container rising 120 seconds and no rinsing). Samples were subjected to erosion cycling, as described above, and each rinsed between cycles. For

spray rinsing, the samples were positioned approximately 5cm away from the tip of a laboratory water bottle containing 100mL of deionised water and sprayed for 30 seconds. For bath rinsing, the samples were fully immersed in a container, filled with 100mL of deionised water, and agitated with an orbital shaker at 60rpm for either 30 or 120 seconds. For no rinsing, after each cycle the samples were removed from the acidic solution for 30 seconds and then immediately re-immersed in the acidic solution. After the final cycle the specimens were spray rinsed.

4.3.5 Storage

The enamel samples were stored differently prior to the erosive challenge. For 'dry' samples each group were stored dry over a 24-hour period at room temperature. For '1 and 24 hour storage' the samples were fully immersed, in 8mL of deionised water for either one or 24 hours, at room temperature. Following storage, specimens were rinsed with deionised water and subjected to the erosive cycling.

4.3.6 Ultrasonication

The effect of ultrasonication after polishing was investigated by subjecting a group of samples to erosion cycling following polishing without any further intervention (no ultrasonication). Following polishing, another group of samples were placed in a weighing boat with 80mL of deionised water and ultrasonicated (Nusonics GP-70, T310) at 60Hz for 15 minutes after which they were rinsed with deionised water and allowed to dry (ultrasonicated). The samples were then eroded as above.

4.3.7 Volume

The effect of the volume of solution into which the enamel samples were immersed was investigated with two groups (80 or 100mL of solution) and subjected to the standard erosion cycling. A large plastic weighing boat (Max volume =330mL, 105 x 105 x 25mm) was used for 80 and 100 mL groups.

4.3.8 Position of Sample

The effect of positioning the sample within the container was investigated with the enamel samples facing upwards or downwards into the solution. Those facing upwards had the reference tape visible to the naked eye, whilst the downwards samples had the tape facing the bottom of the container. The samples were then eroded with the same cycling as above.

4.3.9 Measurements

Profilometry and Knoop microhardness measurements were performed for all experiments as described in sections 2.4.2 and 2.4.3 respectively. For the step height extraction, MountainsMap® was used. Baseline microhardness values were obtained for each specimen and any specimens falling outside a range of Knoop hardness 340H_K ±50 was rejected.

4.3.10 Sample Size Calculation

The sample size calculation was based on 2 way ANOVA for testing mean step height. Assuming an effect size of 0.4 and 80% power the study required a total sample size of 73 to test the significant difference between speed and agitation and interaction at 5% level using a 2 tail test. The power calculation was carried out using gpower3.1.5. Hence it was decided to have at least 10 samples for each combination. Considering this as the standard, for all other measures, this was used.

4.4 Statistical Analysis

Linear models were used to test the significant difference (between the difference measures) between different categories.

The microhardness data did not follow normal distribution so the data was square transformed and the transformed data was used for the analysis.

4.5 Results

4.5.1 Tooth Surface/Type

Figure 32a shows that the buccal surfaces of the molars had a MSH of 6.5µm (±0.8) and for the premolars it was 7.3µm (±1.0). The lingual surfaces of the molars had a MSH of 7.7µm (±1.8) and premolars 8.2µm (±1.6) respectively, which was not significant ($p=0.152$). The premolar teeth produced a MSH of 7.8µm (±1.4) and the molar was 7.1µm (±1.5). Figure 32b shows KHC for buccal and lingual surfaces of the molar was 131.6H_K (±16.3) and 166.3H_K (±18.2) which was significant ($p<0.05$) and for buccal and lingual surfaces of the premolar it was 179.6H_K (±20.0) and 193.2H_K (±20.1) which was also significant ($p<0.05$).

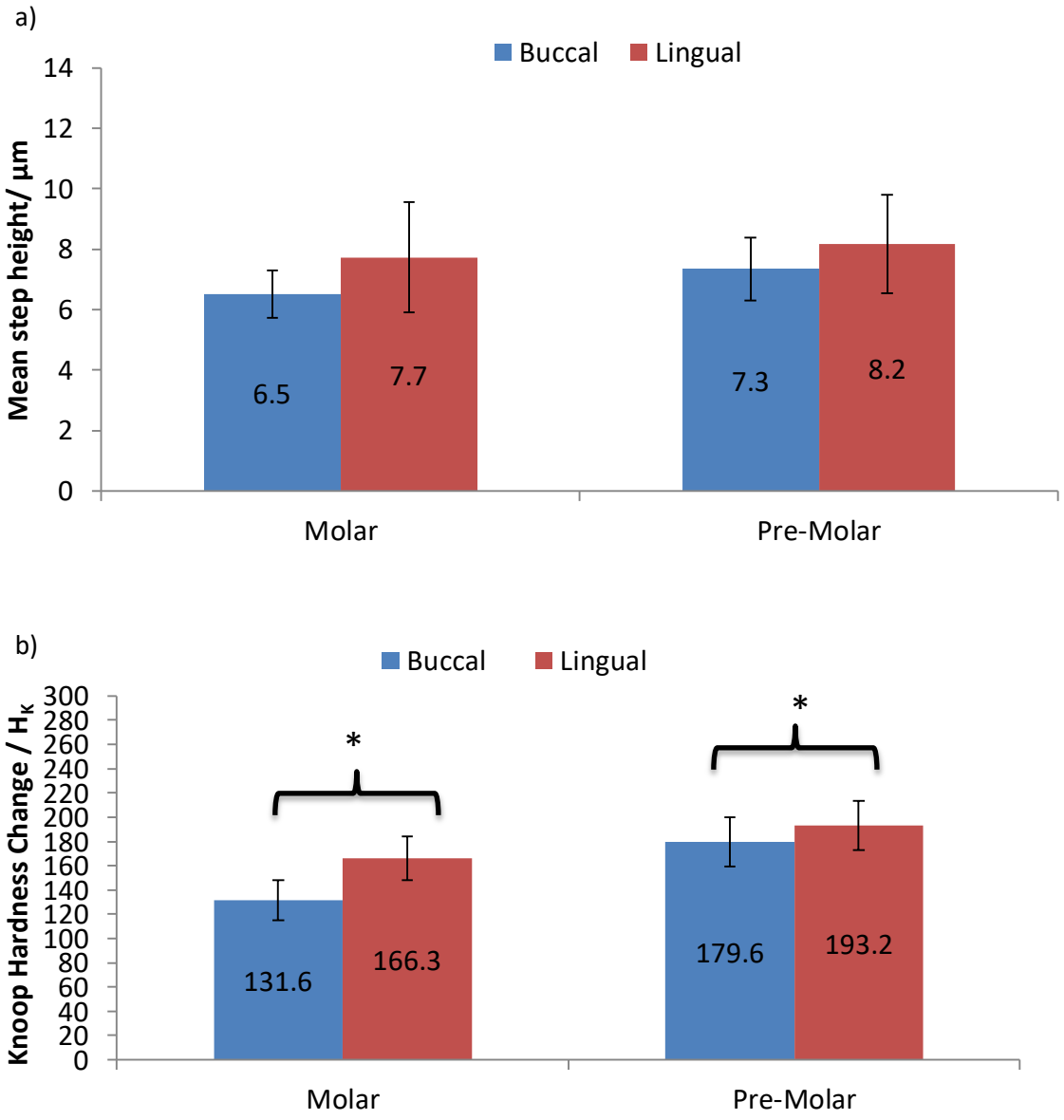

Figure 32 a) Mean step height (µm) with standard deviation b) Knoop microhardness change (H_K) with standard deviation for effect of molar v premolar teeth and buccal v lingual surfaces on *in vitro* erosion. * = statistically significant ($p<0.05$)

4.5.2 Ultrasonication

Figure 33a shows the MSH with and without ultrasonication was 6.6µm (±0.7) and 8.6µm (±1.2) respectively. Figure 33b shows the KHC with and without ultrasonication was 193.0H_K (±14.4) and 232.6H_K (±22.5) respectively. There was a significant difference for both the MSH ($p< 0.05$) and KHC ($p<0.05$).

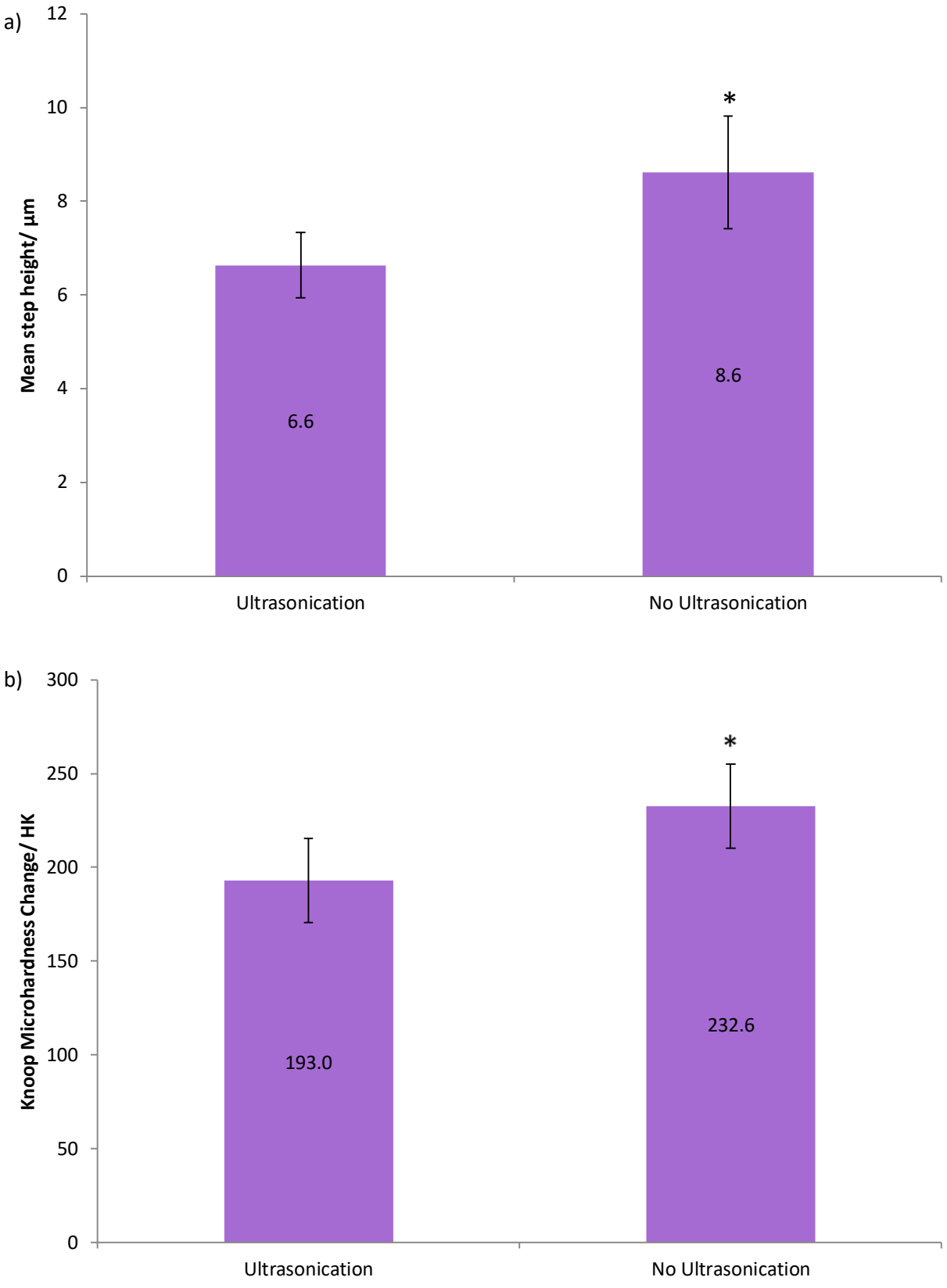

Figure 33 a) Mean step height (µm) with standard deviation b) Knoop microhardness change (loss) (H_K) with standard deviation for effect of a ultrasonication on *in vitro* erosion. * = statistically significant compared to ultrasonication ($p<0.05$)

4.5.3 Storage

Figure 34 a and b show the MSH and KHC with standard deviations for the storage experiment. Storage in deionised water produced less MSH and KHC, compared to the dry samples and this was significant for both MSH (p=0.049) and KHC (p<0.05). The MSH for dry, 1 and 24 hours storage were 7.5µm (±1.), 6.5µm (±0.7) and 6.8µm (±0.8) respectively. The KHC for dry, 1 and 24 hour storage were 212.6H_K (±7.5), 188.2H_K (±18.9) and 186.8H_K (±15.2) respectively.

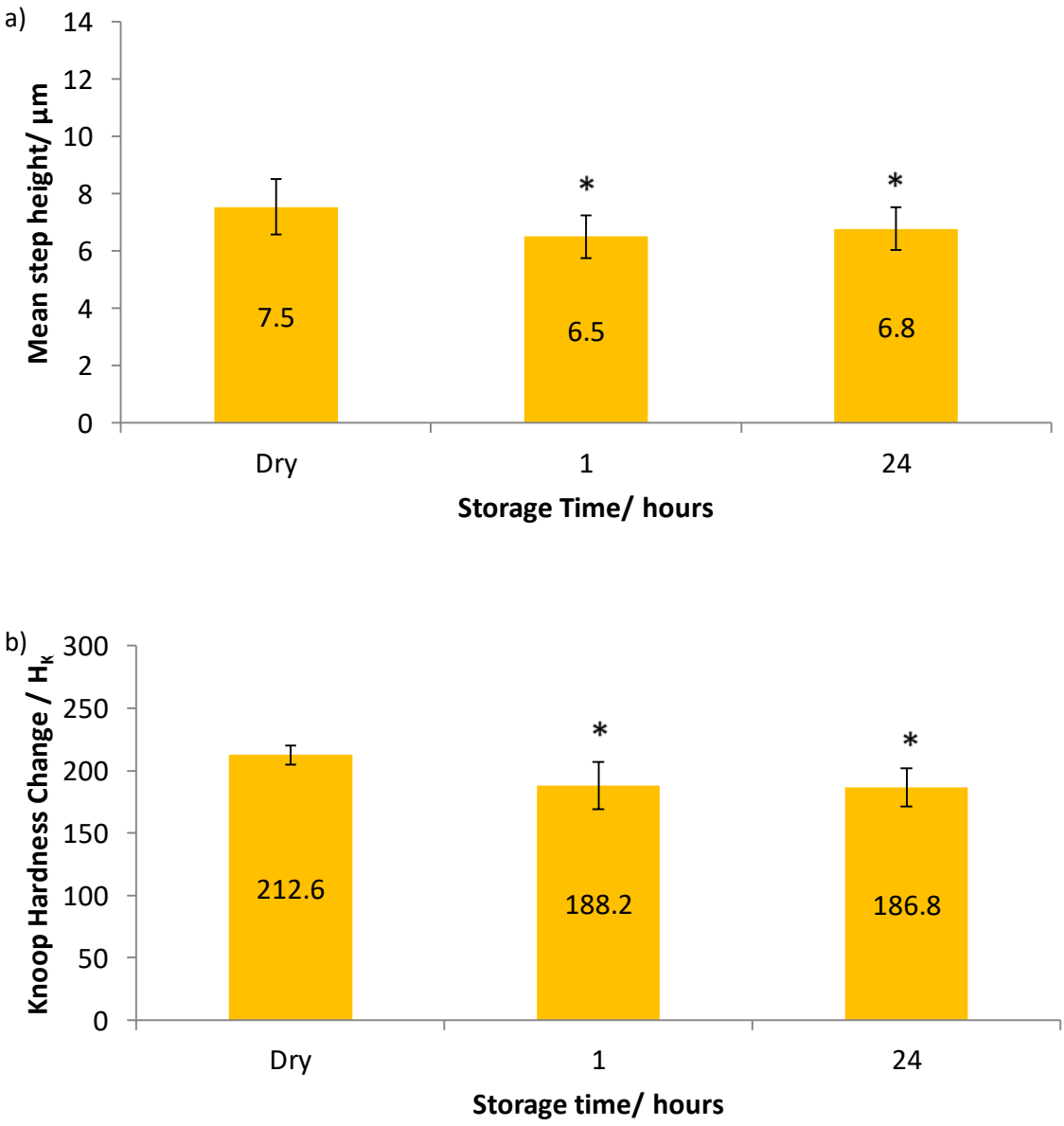

Figure 34 a) Mean step height (µm) with standard deviation b) Knoop microhardness change (H_K) with standard deviation for effect of dry, 1 hour and 24 hour storage in deionsed water prior to an *in vitro* erosive challenge. * = statistically significant compared to dry (p<0.05)

4.5.4 Agitation and speed

Figure 35a shows the MSH with standard deviation for the agitation and speed experiment. The MSH for the control group was 1.0µm (±0.2). Across all groups the highest MSH was 11.7µm (±0.9) for See-saw at 70rpm and the lowest was 2.8µm (±1.1) for the Orbital at 30rpm. For all agitation types, increasing the speed (rpm) increased the MSH. See-saw agitation produced the highest MSH at all speeds compared to Orbital and Gyro and this difference was not significant ($p=0.185$). At the lower speeds, 30 and 40rpm, Orbital agitation produced less MSH than Gyro, 2.8µm (±1.1) and 5.1µm (±0.6) for Orbital compared to 3.9µm (±0.9) and 6.0µm (±1.0) for Gyro. At the higher speeds, 60 and 70rpm, this is reversed and Orbital agitation produced a higher MSH compared to Gyro, 7.5µm (±1.0) and 8.4µm (±1.0) for Orbital compared to 7.3µm (±1.1) and 7.9µm (±1.7) for Gyro. Compared to the control, all groups produced a significantly higher MSH ($p<0.001$).

Figure 35b shows the KHC with standard deviation for the agitation and speed experiment. The KHC for the control group was 87.7H_K (7.2). Across all groups, the highest change was 243.0H_K (±7.3) for See-saw at 70rpm and the lowest was 189.5H_K (±18.7) for Orbital at 30rpm. For all agitation types, increasing the speed increases the KHC. There was little difference in the KHC and no significant difference ($p=0.496$) between the three agitation types. See-saw agitation produced a slightly higher change compared to both Orbital and Gyro. At 30rpm Orbital produced a lower KHC, 189.5H_K (±18.7) compared to Gyro 200.5H_K (±6.2). However, at 40rpm Orbital produced a higher change 212.8H_K (±11.2) compared to Gyro 208.8H_K (±10.1). At 60 and 70rpm the difference was negligible. There was no significant differences compared to the control ($p=0.954$) for KHC.

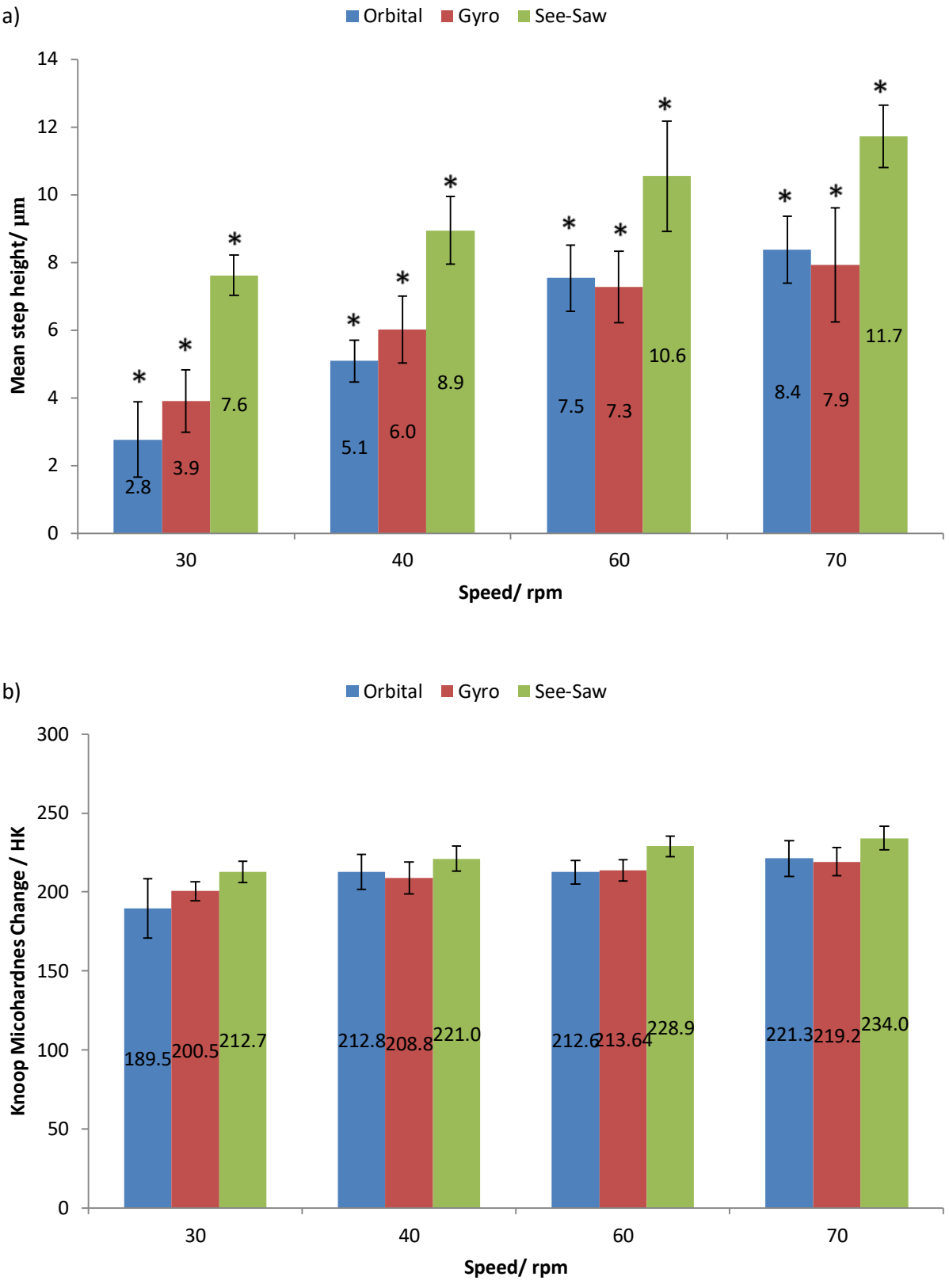

Figure 35 a) Mean step height (µm) with standard deviation b) Knoop microhardness change (H_K) with standard deviation for effect of Orbital, Gryo and See-saw agitaton and 30,40, 60 and 70 rpm on *in vitro* erosion. *= statistically significant compared to the control (p<0.001)

4.5.5 Rinsing

Figure 36a and b show the MSH and KHC with standard deviations for the rinsing experiment. A lower MSH was seen for no rinsing and little difference was seen between the different rinsing types. The mean step height for “no rinsing” was 5.3µm (±0.6), which was significantly different ($p<0.05$) compared to spray rinsing for 30 seconds, 8.6µm (±1.2), container rinsing for 30 seconds 9.3µm (±1.1) and container rinsing for 120 seconds, 8.8µm (±1.4). The KHC for “no rinsing” was 207.4H_K (±11.3) which was significantly different ($p<0.05$) compared to, spray rinse 30, 218.8H_K (±12.0), container rinse 30, 213.0H_K (±10.3) and container rinse 120 223.9H_K (±16.1). No significant differences were observed when comparing spray rinse 30 to bath rinse 30 ($p=0.194$) and bath rinse 120 ($p=0.335$) and bath rinse 30 to bath rinse 120 ($p=0.732$) for MSH. No significant differences were observed for KHC when comparing spray rinse 30 to bath rinse 30 ($p=0.21$) and bath rinse 120 ($p=0.332$) and comparing bath rinse 30 to bath rinse 120 ($p=0.335$).

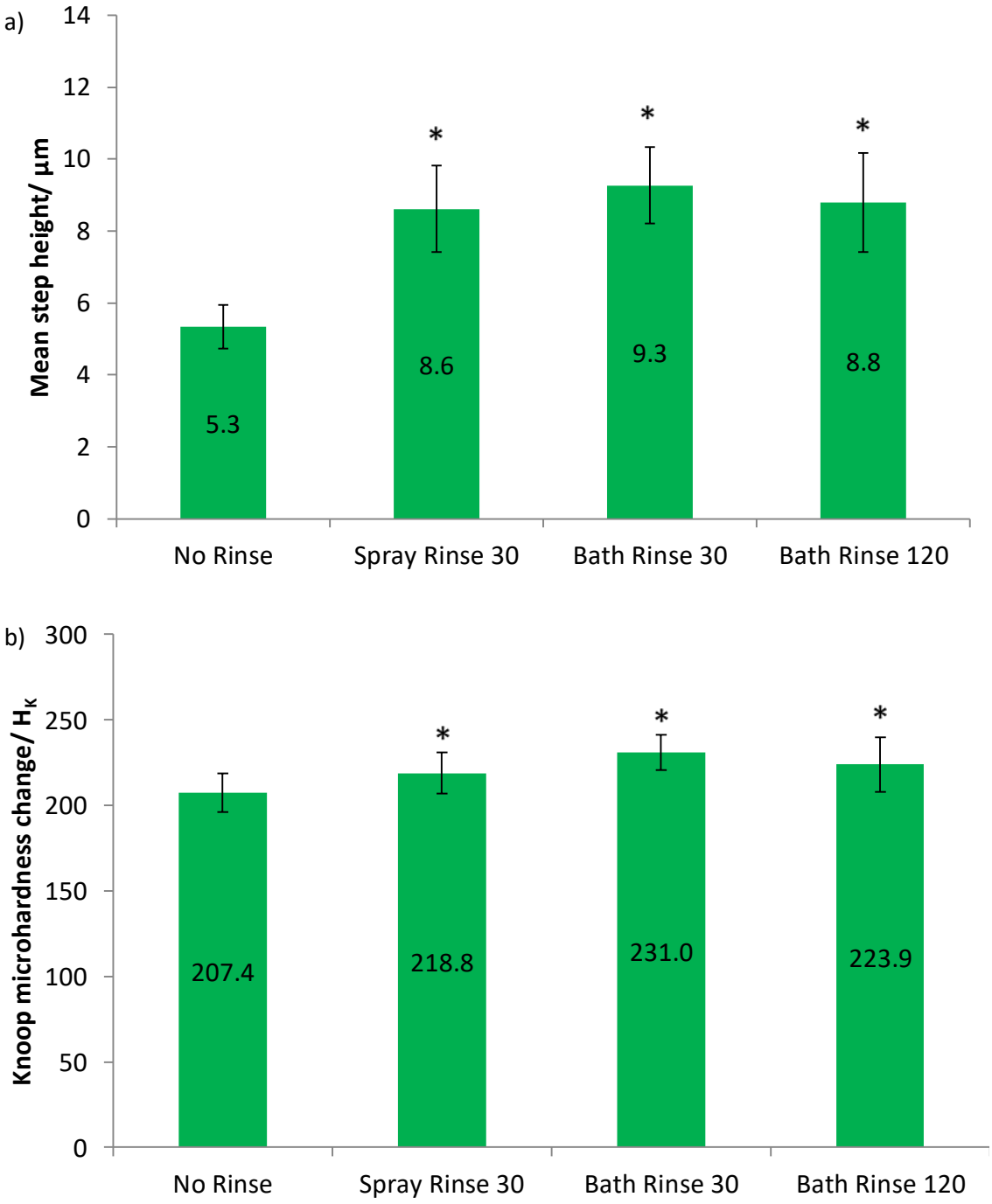

Figure 36 a) Mean step height (µm) with standard deviation b) Knoop microhardness change (H_K) with standard deviation for effect of no rinsing, spray rinse 30 seconds, container rinse 30 seconds and container rinse 120 seconds on *in vitro* erosion. * = statistically significant compared to 'no rinse' ($p<0.05$)

4.5.6 Volume

Figure 37a and b show the MSH and KHC with standard deviations for the volume experiment. Increasing the volume produced a decrease in the MSH and KHC. For MSH the data for 80mL was 7.5µm (±1.0) and for 100mL was 7.1µm (±0.9). For the KHC, 80mL was 212.6H_K (±9.6) and for 100mL was 181.4H_K (±15.1). There was a non-significant difference between 80mL and 100mL for the MSH (p=0.313) but a significant difference for the KHC (p<0.05).

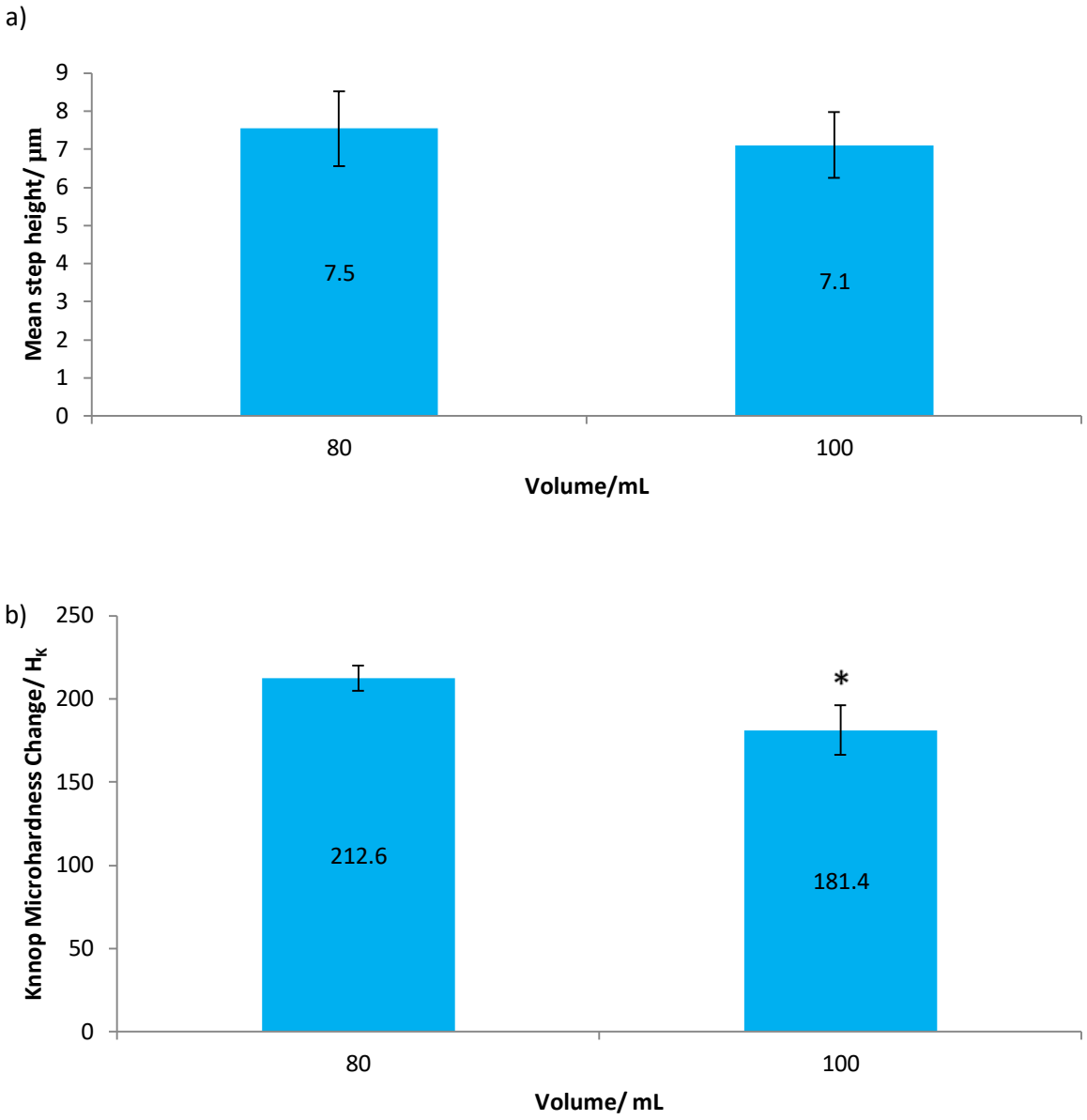

Figure 37 a) Mean step height (µm) with standard deviation b) Knoop microhardness change (loss) (H_K)with standard deviation for effect of 80 and 100 mL of erosive solution on *in vitro* erosion. * =statistically significant (p<0.05) compared to 80mL

4.5.7 Position of Sample

Figure 38a and b show the MSH and KHC with standard deviations for the 'position of sample' with the enamel surface facing 'up' out of the solution or facing 'down' into the solution. The enamel surfaces facing 'down' into the solution produced a lower mean step height, 0.9µm (±0.3), compared to that facing 'up', 3.7µm (±0.8). For KHC, the samples facing 'down' produced a lower KHC, 108.1H_K (±29.9) compared to samples facing 'up', 246.4H_K (±59.1). There was a significant difference comparing 'up' and 'down' for the MSH and for the KHC ($p<0.05$).

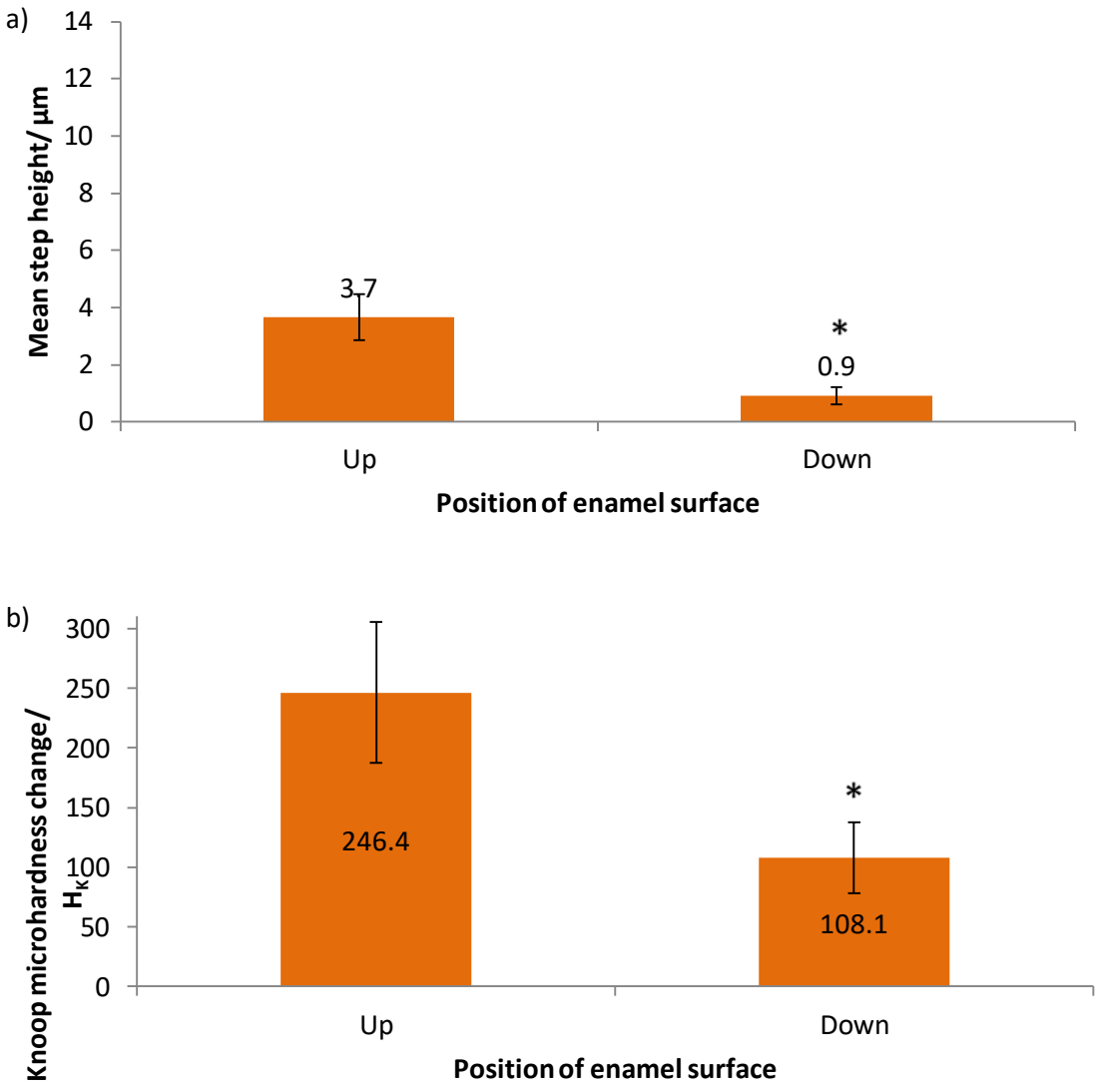

Figure 38 a) Mean step height (µm) and b) Knoop microhardness change (H_K) with standard deviations for effect of the position of the enamel surface in the solution (up or down) on *in vitro* erosion. * = statistically significant compared to sample facing up ($p<0.05$)

4.6 Discussion

Overall, it was observed that the different model variables produced differences in the MSH and KHC, with some having a larger impact than others.

4.6.1 Tooth surface/type

The type of teeth and surface investigated were chosen as these are the most commonly used in *in vitro* studies (Shellis et al. 2011; Young & Tenuta 2011). The buccal and lingual surfaces are also the main surfaces that would come into contact with the food or beverage that is consumed and so these surfaces would be the most applicable when attempting to model tooth erosion *in vitro*. Another advantage is that the buccal and lingual surfaces also contain some of the thickest part of the enamel on the tooth. This allows a polishing procedure to be applied with less risk of exposing dentine. The results showed that there was no statistical difference between the tooth types and surface for profilometry but there was for Knoop microhardness. A possible explanation is that after the erosion cycles, there will be loss of enamel (detected by the profilometer) but also a layer of demineralised softened enamel. Theoretically the profilometric loss of enamel could be the same. However, as the Knoop microhardness measures the extent of the softened enamel left after the profilometric tissue loss, this has the potential to detect differences.

Molar teeth produced less Knoop microhardness change and therefore surface softening than premolar teeth. Buccal surfaces produced less surface softening compared to the lingual surfaces for both the premolar and molars. This could be due to the difference in mineral content between the different teeth and between the different surfaces on the same tooth as shown by (Wong et al. 2004; Egan et al. 2013). If the buccal surfaces and molar teeth had a higher mineral content they would be more resistant to erosion as they would be able to lose more minerals before experiencing bulk tooth surface loss. Also, it is possible that the buccal surfaces and molar teeth could have shorter hydroxyapatite crystals which have shown to be

harder by computer modelling (Lu et al. 2012) and would explain our Knoop microhardness results, therefore making them more resistant to erosion and surface softening. To the author's knowledge there are no studies that have investigated the effects of different polished human tooth surfaces and types on *in vitro* erosion measured with non-contact profilometry and microhardness.

A study by Carvalho and Lussi investigated the effects of human molar and premolars, buccal and lingual surfaces and enamel depth with an *in vitro* initial erosion model. They measured the surface microhardness loss and calcium release to assess the amount of erosion caused (Carvalho & Lussi 2015). They reported no significant differences ($p=0.370$) for Knoop microhardness loss between lingual and buccal surfaces, which differs with these results. Also they showed significantly greater ($p<0.05$) softening in premolars compared to molars whereas our results showed greater softening that was not significantly different. This study shows that there was a difference in the susceptibility of different teeth and surfaces to erosion. Unfortunately there was no profilometric data to compare as Carvalho and Lussi did not include profilometry.

A study by Tucker et al. on natural tooth surfaces showed that buccal surfaces produced less surface loss compared to lingual (Tucker et al. 1998) which supports our results, however, they compared maxillary and mandibular teeth and did not specify the specific teeth used. This would introduce some variation however performing experiments on natural tooth surfaces provides valuable information and is more clinically relevant. The most comparable work was published by Ganss et al. where they compared surfaces of molar teeth that had been polished to 4000 grit and analysed with profilometry (Ganss et al. 2000). Similar to our results, they reported buccal surfaces to be less eroded than lingual surfaces with no significant differences between them.

Several papers offer guidance/opinions on choosing variables such as erosive solutions, time of exposure, measurement systems etc. For *in vitro* studies; all suggest that human enamel should be used and one of the papers suggests that only one type of tooth and surface should be used for a study. However, more evidence is needed to support these statements (Young & Tenuta 2011; Curzon & Hefferren 2001; Shellis et al. 2011).

Based on our evidence, molar teeth would be the preferred choice of tooth for *in vitro* studies as these gave the least variation in the MSH with the best standard deviation than the other groups. Premolars may also be included as no significant difference between the buccal and lingual surfaces was found. For profilometry techniques the tooth type and surface would not influence the outcome but for microhardness selection it is more important.

4.6.2 Ultrasonication

After any polishing protocol debris from the polishing material and other contaminants is formed on the surface (Sanches et al. 2009; Watari 2005). The use of acid to remove the smear layer is contraindicated as it would further erode the surface. An alternative was to clean the samples either with rinsing in tap water or with ultrasonication. Not cleaning samples with ultrasonication produced a MSH of approximately 2µm more than when the samples were subjected to ultrasonication with deionised water and this difference probably represents the thickness of the smear layer. This might also explain why larger Knoop microhardness change was recorded for the ultrasonicated samples. The debris and smear layer probably consisted of debris from the silicon carbide polishing disks and silicon carbide is harder than enamel with a hardness Knoop value of approximately 2000 (Majić et al. 2011).

To the best of this author's knowledge there are no studies that have investigated the effects of ultrasonication on *in vitro* erosion. There are a few studies that investigated the polishing smear layer. Watari estimated a smear layer thickness of approximately 0.5µm created when polishing with alumina emulsion (0.05µm grain size) and approximately 0.27µm when

polishing to 2000 grit (approximately 12µm grain size). My protocol polished to 4000 grit (5µm grain size) and so it could be speculated that a 'polishing smear layer' would be between the two values. But this difference would not account for the step heights observed in this experiment. Watari did not report on the time spent on polishing and force used which might have affected the results (Watari 2005). Jager et al. removed the smear layer from the enamel surface with a solution containing citric acid, KH_2PO_4, $CaCl_2$ and NaN_3. The addition of the salts would have reduced the erosive potential of the solution. However, their study used the calcium release to assess the amount of erosion caused which is not dependent on a flat surface for accurate measurements (Jager et al. 2012).

Using ultrasonication, after polishing, would be recommended as there is a possibility that the polishing debris and other residual dirt could influence the data.

4.6.3 Storage

The effect of storage is effectively a question about hydration status of the enamel. Samples can be stored dry or in deionised water. The 'storage in deionised water' group could be split further into the length stored in the deionised water. One and 24 hours were chosen as convenient storage times. One hour is a convenient time to wait and fits within normal experimental times whereas 24 hours was chosen as it has been used in other studies that store samples wet, in artificial saliva or in humid conditions (Yamashita et al. 2013; Çehreli et al. 2012; Li et al. 2013). Also, any effect of hydration would occur within 24 hours and so a time longer than this would not yield any new information and risk bacterial growth.

Overall there was very little change in the MSH between the different storage conditions. The samples stored in deionised water showed slightly less loss and this difference was statistically significant. The 1 and 24 hour storage data suggested that any effect of hydration occurred within an hour and so storage after this time point is unlikely to affect the enamel.

To this author's knowledge there are no studies that have investigated the effect of storage conditions on enamel before *in vitro* erosion experiments. Shellis et al. reported unpublished data by Lussi and showed that storage in a humid chamber in the presence of thymol crystals for several days (no exact figure is given) had no effect on the micro or nanohardness as was observed in this study (Shellis et al. 2011). However, it is not stated whether the storage conditions were applied before the experiment was carried out or after. Attin et al. investigated storage conditions of enamel after an erosive challenge and concluded that step height measurement of enamel was not affected by storage in wet or dry conditions or after several de/rehydration cycles (Attin et al. 2009).

Using samples that have been stored dry makes standardisation, replication and measurement more convenient.

4.6.4 Agitation and speed

The agitation equipment used in this study was chosen as they are commercially available and often found in laboratories. Each offered a different range of motions, which created different fluid motion. They were all made by the same manufacturer, which meant standardisation of the parts, controls, calibration of the speed and manufacturing process. The speed of 70rpm was chosen as this was the maximum speed for the See-saw and Gyro shakers and 30rpm was chosen as this was the lowest speed that could be used with the Orbital shaker. 40 and 60rpm were chosen as steady increases in the speed to 70rpm so that a picture of gradual increases in the speed could be observed.

Increasing the speed increased the MSH for all agitation types and this was expected and has been shown by other authors (Eisenburger & Addy 2003; Attin et al. 2012). Increases in speed increased the fluid motion and so increased the rate of clearance of the dissolution products over the sample. As the speed increased, the dissolution products over the surface of the enamel were removed quicker, maintaining a concentration gradient and therefore the erosive

process. This replenished the surface with hydrogen ions and acid molecules, which helped to maintain the erosive process. At higher speeds the solution would have had more energy and the motion of the liquid alone through frictional forces could potentially dislodge and remove more of the softened enamel. However, a study by Shellis et al. investigating different flow rates and using SEM images concluded that even at a higher flow rate; the velocity of the solution flow was not high enough to remove partially dissolved crystals. Their apparatus and protocol were different to ours so we cannot say for certain that our conditions would not have physically removed the softened enamel (Shellis et al. 2005).

The Orbital motion produced the lowest MSH and the See-saw rocker produced the highest. The 3D Gyro rocker produced a motion that is described by the company as 'gently swirling' whereas the Orbital shakers motion was a 'swirling' action. It might have been expected that the Gyro rocker would have produced a lower MSH compared to the Orbital as the motion was gentler than the Orbital; however this was only partly the case. The See-saw produced a 'wave motion' that gave a much higher MSH. It could be that the 'wave' motion is better at removing the dissolution products and replenishing the surface with acid molecules, followed by the Gyro and then the Orbital. The manufacturers description of the See-saw agitation is that it is ideal for 'washing' samples, the Orbital agitation is ideal for 'aeration' and the Gyro agitation is ideal for cell culturing, staining and de-staining (which in our case is irrelevant).

Previous investigations on flow rate (Attin et al. 2012; Eisenburger & Addy 2003; Shellis et al. 2005) have shown that there is an increase in erosion with increasing flow rate, which is confirmed by this work. However, the apparatus used in those studies were custom made, whereas the work presented here used basic agitation methods found in most laboratories. Most authors report that agitation increases step height loss in laboratory investigations (Attin et al. 2012; Eisenburger & Addy 2003; Shellis et al. 2005). It is unknown how well the agitation methods used here represent the clinical situation.

A recommendation for speed and type of agitation is difficult as the choice depends on the container and amount of volume of solution. When agitating samples, care must be taken to prevent spilling of the solution and this is determined by the container, speed and volume of solution. However certain agitation types can be more aggressive. The See-saw and Orbital was more aggressive than the Gyro and during the experimental procedure the solution was on the edge of spilling over. Any decision on the choice of agitation depends on the apparatus available and which best compliments the container and volume of solution.

4.6.5 Rinsing

The three different rinsing types were chosen as these covered the main options. The samples were agitated using the same parameters as described (orbital shaker, 60rpm). The timings for the rinsing were chosen based on common practice (see Chapter 3) and the 30 seconds used in previous experiments. For comparison, 120 seconds was selected as the upper scale limit for ensuring the samples were properly rinsed. Pilot work revealed that after applying a similar pressure on the spray bottle for 30 seconds, 80-100mL of deionised water was consistently dispensed. For simplicity and standardisation 100mL was chosen as the amount of water to use.

One would have expected that the 'no rinsing' would produce a higher step height loss, as the erosive process was not being stopped by dilution/rinsing, however this did not occur. An explanation could be that the Nernst layer remains at the surface of the enamel, steadily increasing in thickness (Attin et al. 2012) and therefore reducing the amount of calcium and phosphate ions diffusing into the solution. As the Nernst layer becomes more saturated in the tooth minerals this would affect the diffusion gradient by lowering it with respect to the calcium and phosphate ions. As rinsing was the only altered variable in this experiment, this data suggests that the motion of the fluid under agitation with an Orbital shaker at 60rpm or the force of the spray rinsing is not enough to interrupt this layer.

The time spent rinsing did not statistically influence the amount of step height loss and had a similar effect on the amount of erosion caused. From these results and under these conditions, there was no difference between the rinsing method/time but choosing to rinse or not rinse samples after an *in vitro* challenge would significantly affect the level of erosion. To the author's knowledge, there have been no studies investigating the effects of different types and times of rinsing. Very little detail is provided on the method used to rinse the samples, however the important message is whether the samples have been rinsed or not.

Using a container to rinse the samples is recommended by the author as it increases the level of standardisation.

4.6.6 Volume

The greatest volume that could be placed in a large container with 10 samples without spilling over during the agitation was 100mL, and the lowest volume was 80mL. Increasing the volume from 80 to 100mL gave a decrease in the MSH. One would have expected that an increase in the volume (and therefore the volume per sample) would therefore increase the MSH as there would be more acid molecules available. This result revealed that fluid motion may have a greater impact on the amount of erosion caused compared to volume of acid *in vitro*. A visual observation of the solution was that at the larger volume the solution appeared to move less (it was less aggressive). At these volumes, the amount of acid molecules had less of an effect on the amount of erosion caused *in vitro* than the motion of the solution.

Other studies investigating volume have done so in terms of flow rate, measuring how much solution is passing over the sample. It has been shown that larger volumes will increase the amount of erosion caused (Shellis et al. 2005). Most *in vitro* studies standardise the volume of erosive solution. The effect of speed has been shown previously by other researchers to increase the amount of erosion as explained in section 4.6.4.

It is difficult to recommend a volume of erosive solution, as it is dependent on a variety of factors. The most important point is that the volume needs to be sufficient enough to cover the sample. The amount required to cover the samples will be dependent on the size and number of the samples and the size/shape of the container. Also the solution should not spill out of the container. Choosing a container size depends mainly on the number of samples and amount of erosive solution. The container has to be large enough to fit the number of samples and volume of erosive solution of the experiment.

4.6.7 Position of sample

For *in vitro* studies, the enamel surface must be fully immersed in the experimental solution to ensure that the surface is in constant contact with the solution. There are several ways to implement this but using the equipment described above the most practical are that they be fully submersed in the bottom of a container with the enamel surface facing up or that they could be floating on the top with the enamel surface facing down. Either way, the enamel surface is in constant contact with the experimental solution when immersed.

When the sample was facing up, the step height change was greater and this could be due to the better clearance of dissolution products and replenishment of the hydrogen ions compared to the samples facing down. The agitation of the solution was the same in both instances (Orbital and 60rpm) but it appears that the enamel surfaces have experienced different flows based on how they are positioned in the solution. There is one study that reported placing a sample 'upside down' in an acid and found that this produced a lower MSH compared to facing up, however the difference was not significant and this study did not agitate the samples (Paepegaey et al. 2013).

Facing the samples up in the solution allowed greater control during the experimental procedure.

4.7 Summary

The eight variables tested can be grouped together; three were related to sample preparation (tooth surface and type, ultrasonication and storage) and five to fluid motion (agitation, speed, rinsing, volume and position of sample). They all showed an effect on the measurable outcome on *in vitro* erosion and reject the null hypotheses. The overall conclusion from this work is that it highlights the importance of standardisation of the protocols. This set of experiments show that small changes to variables can affect the outcome. The work also highlights the importance of accurate and detailed reporting of the method and materials. The method sections of papers need to provide sufficient detail of the set-up and variables, something which is often lacking in some studies. The decision on measurement technique will influence the study design as several of the investigations showed a significant difference for only one of the measurement techniques within the same study.

Chapter 5. Sodium and stannous fluoride application time

5.1 Introduction

This chapter aimed to use the experience gained from the previous Chapters to design and conduct an investigation on the effect of sodium and stannous fluoride and the timing of the application. Previous work has shown that stannous and sodium fluorides have the ability to prevent dental erosion (Willumsen et al. 2004; Eversole et al. 2014; Carvalho & Lussi 2014). However, both fluorides have differing modes of action that could behave differently under the same conditions. Fluorides can be applied before or after an erosive challenge. The enamel surface and subsurface may react differently depending on whether an acid attack occurred prior to or after immersion in fluoride. The role of the fluoride ion pre or post acid exposure would be to prevent demineralisation and promote remineralisation; however which role is more dominant may depend upon the metal ion. It would be beneficial to know whether there is a difference in fluoride efficacy depending on the timing of application. The experiments in this Chapter used standard formulated fluorides at mouth rinse concentrations and applied them either before or after immersion in citric acid.

5.2 Aims, Objectives and Hypothesis

5.2.1 Aims

The aim was to investigate the effect of stannous and sodium fluoride solutions and the timing of their application on *in vitro* erosion.

5.2.2 Objectives

- To use the knowledge gained from the previous work to conduct an erosion model to investigate the difference in erosion protection from sodium and stannous fluoride solutions at a commercial mouth rinse concentrations (225ppm)

- To investigate whether application before or after an erosive challenge has any difference in the level of erosion

5.2.3 Null Hypotheses

- There is no difference in the step height or microhardness change between sodium and stannous fluoride

- There is no difference in the step height or microhardness change when applying fluoride before or after an erosive challenge

5.3 Materials and Methods

The general principles from those described in Chapter 2 were followed with the exception of outcomes described in Chapter 4. Enamel sections from the buccal surfaces of molar teeth were sectioned using a Buehler Isomet 1000 precision saw with an Extex diamond wafering blade, mounted with cold cure acrylic resin and then polished to 4000 FEPA grit number using a Buehler Metaserv 3000 variable speed grinder-polisher and Vector™ LC power head. They were randomly divided into four groups of 10 sections. Immediately after polishing the sections were ultra-sonicated (Nusonics GP-70, T310) at 60Hz for 15 minutes in 80mL of deionised water to remove remaining polishing debris and dirt. Citric acid at 0.02M concentration and adjusted to pH 3.2 was used as the erosive solution, made by the procedure described in (Chapter 2, section 2.2).

5.3.1 Fluoride solutions

Sodium fluoride 99% (0.49g) (Alfa Aesar, lot# 10148378, product code-A13019) and stannous fluoride 99% (0.93g) (Sigma Aldrich, lot# MKBP3104V, product code-100156526) were added to 1 litre of deionised water in volumetric flasks to make 225ppm solutions. The solutions were mixed with a magnetic stirrer bar until the solid was fully dissolved. The fluoride solutions were used on the day that they were made.

5.3.2 Cycling procedure

Samples were placed in the solution with the enamel surface facing 'up' and agitated in 80mL of citric acid at 0.02M for 10 minutes using an orbital shaker (60rpm). For erosion followed by fluoride application (Figure 39), the samples were rinsed in 100mL deionised water that was agitated with an Orbital shaker at 60rpm for 2 minutes. During fluoride application, the samples were immersed in 80mL of the appropriate fluoride solution and agitated with an Orbital shaker at 60rpm for 1 minute. During the wait time, the samples were placed unstirred in 100mL of deionised water. The fluoride solutions and the deionised water were replaced for each cycle. For fluoride followed by erosion (Figure 39), the same cycling procedure was followed as above, but with the fluoride application as the starting point. The negative control group followed the sample cycling procedure with deionised water in place of the fluoride.

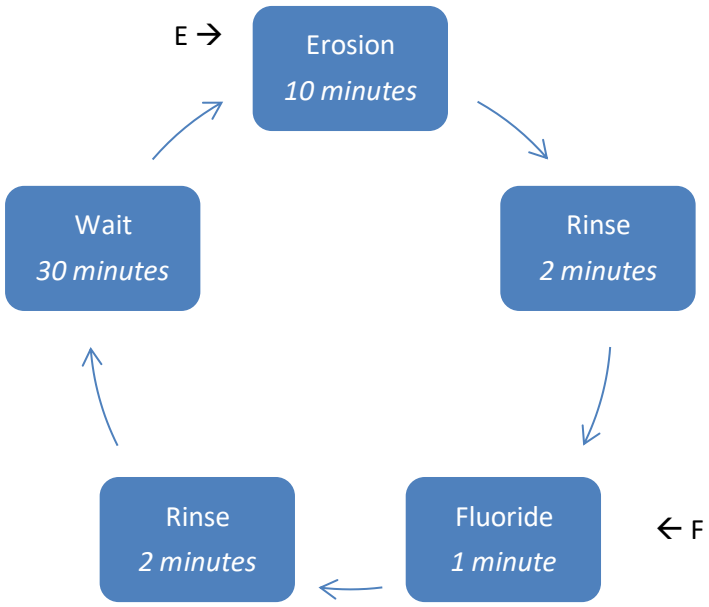

Figure 39 Cycling procedure for fluoride experiment. With 'E' showing the start point for erosion followed by fluoride application and 'F' showing the start point for fluoride application followed by erosion

5.3.3 Measurements

Profilometry and Knoop microhardness measurements were performed for all experiments as described in sections 2.4.2 and 2.4.3 respectively. For the step height extraction, MountainsMap® was used. Knoop microhardness change was calculated by subtracting the worn hardness away from the reference hardness.

5.3.4 Sample Size Calculation

The sample size calculation for this measure was based on 2 way ANOVA for testing mean step height. Assuming an effect size of 0.4 and 80% power the study required a total sample size of 52 to test the significant difference between fluoride and time and interaction at 5% level using a 2 tail test. The power calculation was carried out using gpower3.1.5.

5.4 Statistical Analysis

Linear models were used to test the significant difference (between the fluorides and application times) between different categories. P values are to be adjusted for multiple comparisons.

5.5 Results

Figure 40a and b show the MSH and KHC with standard deviations for the application of sodium and stannous fluoride before and after an erosive challenge. The control group produced a MSH of 12.6µm (±1.2) and KHC of 118.9H_K (±15.4). The stannous fluoride solution produced lower MSH values compared to sodium fluoride ($p<0.05$). For sodium fluoride, application after the erosive challenge produced a MSH of 12.3µm (±0.9) and application before the erosive challenge the MSH was 12.6µm (±1.4) and this difference was not statistically significant ($p=0.361$). For stannous fluoride, application after the erosive challenge produced a MSH of 7.5µm (±0.8) and before the erosive challenge, it was 6.5µm (±1.2) and this difference was statistically significant ($p<0.05$).

Stannous fluoride immersed samples produced larger KHC values compared to the sodium fluoride and this was statistically significant ($p=0.049$). However, there was no statistical difference in the KHC in the timing of application of fluoride. For sodium fluoride, application after an erosive challenge produced a KHC of 133.7H_K (±12.9) and before the erosive challenge it was 135.0H_K (±10.4) and this difference was not statistically significant. For stannous fluoride, application after an erosive challenge produced a KHC of 171.8H_K (±22.1) and before the erosive challenge it was 163.7H_K (±21.5) and this difference was not statistically significant ($p=0.195$).

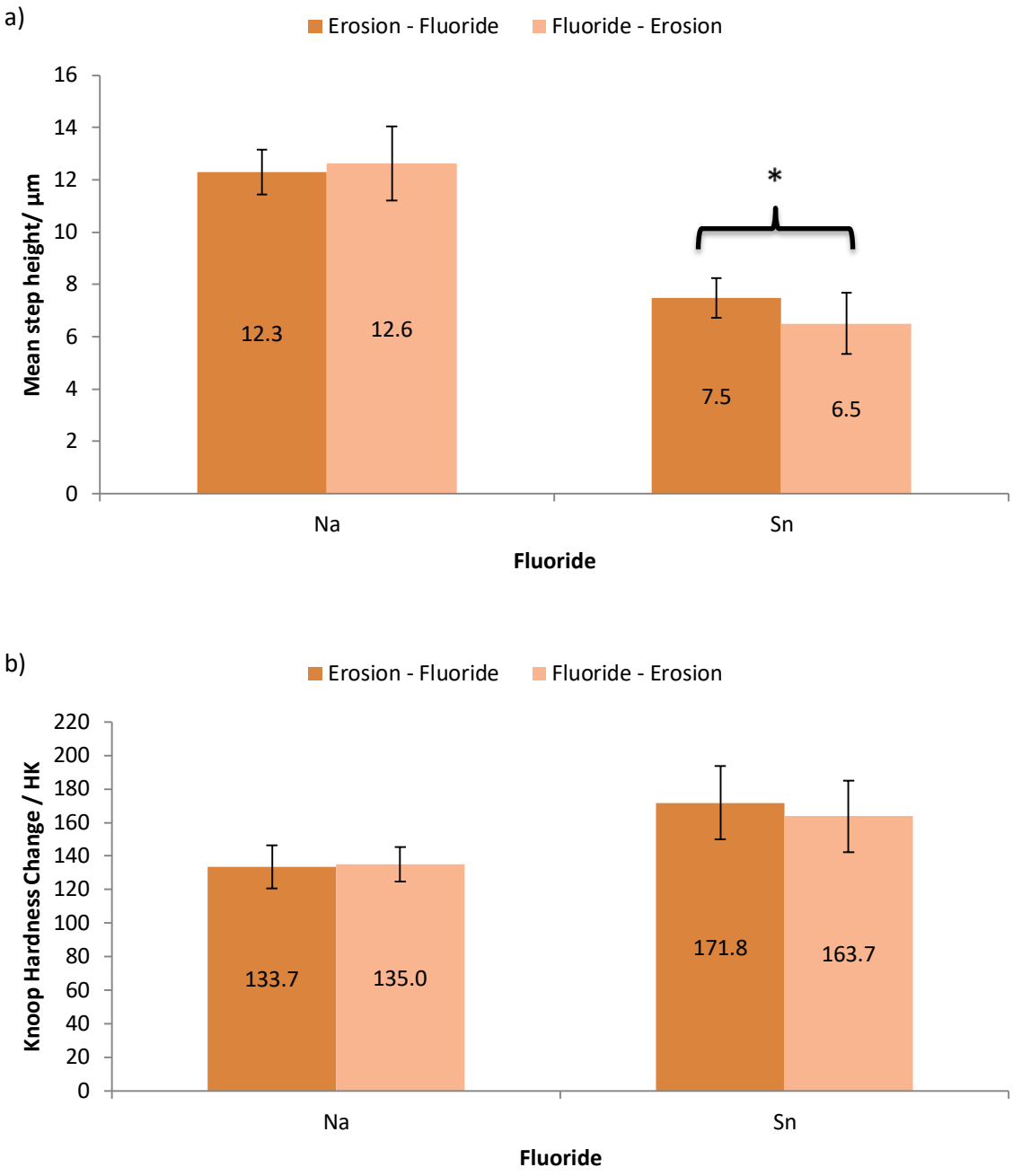

Figure 40 a) Mean step height (µm) and b) Knoop microhardness change (H_K) with standard deviation for stannous and sodium fluoride application time experiment. Orange bars represent erosion followed by fluoride and pink bars represent fluoride followed by erosion. * = statistically significant ($p<0.05$)

5.6 Discussion

The results showed that under these experimental conditions, stannous fluoride produced significantly less MSH compared to sodium fluoride and therefore it could be said that it provided better protection.

Sodium and stannous fluoride were selected as there are differences in their chemistry that could alter their efficacy depending upon the environment. Sodium fluoride is a monovalent compound able to donate one fluoride ion per molecule and stannous fluoride is a polyvalent compound capable of donating more than one fluoride per molecule. The application of the fluoride solution before or after an erosive challenge was undertaken to investigate whether the interaction between the metal ion and enamel surface/subsurface, under different surface conditions was important in how they protect against erosion. The concentrations of fluoride (225ppm) was chosen to represent commercial mouth rinses and has been used in other studies (Takagi et al. 2001; Gracia et al. 2010; Favretto et al. 2013). The wait time was introduced into the protocol as it allowed for a greater representation of the clinical situation. Instructions for mouth rinses often advise waiting a certain number of minutes before eating or drinking with a typical time being between 30 minutes to 1 hour.

As the concentration of the fluoride ion within the solutions was the same, the same amount of fluoride should have been available in the solution. This would then suggest that the metal ion influenced the effectiveness of the fluorides availability or formation of a fluoride containing protective layer. The Knoop microhardness change was higher for the stannous fluoride than the sodium fluoride. This change suggests that the sub-surface might be softer with stannous fluoride and although less profilometric enamel loss had occurred, this might imply that the enamel matrix remained intact. Therefore it was softer but had the potential for remineralisation. White et al. investigated the dose response of sodium fluoride on hydroxyapatite dissolution and showed that after 30 seconds application, a concentration of

112ppm was the limit of the protective effect (White et al. 2012). Higher concentrations should not provide more protection and so they postulated that the substitution of hydroxide ions in the enamel structure by fluoride ions reached a maximum at 112ppm. The study in this Chapter only investigated one concentration (225ppm) and it would be beneficial to investigate concentrations higher and lower to explore a dose response effect.

Baig et al. studied various concentrations of stannous and sodium fluorides on enamel erosion and found that stannous fluoride provided better protection than sodium fluoride at all concentrations, with the closest to our study being 200ppm (Baig et al. 2014). Other studies have also shown stannous fluoride to provide better protection compared to sodium fluoride (Huysmans et al. 2011; Faller et al. 2014; Venasakulchai et al. 2010) and support our findings. However, one study observed a conflicting result (Barlow 2009) but used microhardness to evaluate change and used a product designed as an anti-tartar agent that might influence the data.

Takagia and Chowa studied the effect of a two-component fluoride rinse system on the effect on *in vitro* erosion. They used a low concentration of sodium fluoride (250ppm) and showed that at that concentration only 6% of the fluoride was deposited on the surface, which was 0.21 $\mu g/cm^2$ of deposition. This might explain the relatively ineffective protection against erosion however unfortunately there is no stannous fluoride data to compare it with (Takagi et al. 2001).

Willumsen et al. compared the effect of stannous and sodium fluoride solutions applied before an erosive challenge, measuring calcium release. They used different concentrations of stannous (0.4%) and sodium fluoride (2%) and experimental protocols (18 hour fluoride exposure times) using hydrochloric acid as the erosive agent. They showed that stannous fluoride provided better protection than sodium fluoride in an acid environment and that stannous compounds were present on the enamel surface both before and after an acid

exposure but not sodium compounds (Willumsen et al. 2004). Our study supports their findings but with relatively more clinically relevant times and with comparable fluoride solutions.

A study by Ganss et al. appeared to provide a better comparison with our study as they investigated the effects of sodium and stannous fluoride at 225ppm on *in vitro* erosion with citric acid; however they used a lower pH citric acid solution and included a remineralisation stage. They also showed that stannous fluoride provided better protection than the sodium fluoride (3.8μm (±14.4) vs -13.2μm (±21.7)) and speculated that the sodium ion contributed to the dissolution behaviour of CaF_2-like layers, which would partly explain why the sodium fluoride solutions provide less protection than the stannous fluoride (Ganss et al. 2008). It was also suggested that remineralisation was ineffective until the sodium salt had been washed away, meaning that the wait time and rinsing in the experiment were important. The CaF_2 layer is poorly soluble and releases small amounts of fluoride, meaning that there is less fluoride available for remineralisation. If this is the main mechanism by which sodium fluoride provides its protection then it could explain the findings.

The results of applying the fluoride before and after the erosive challenge showed different results for each fluoride. For sodium fluoride there was little change but for stannous fluoride there was a statistically lower MSH when applying the fluoride before (6.5μm (±1.2)) the erosive challenge compared to after (7.5μm (±0.8)). For the Knoop microhardness change, there was effectively no difference in applying before or after for both fluorides. The higher MSH seen when applying the stannous fluoride after an erosive challenge suggests that an eroded enamel surface is less effective on the uptake of the stannous fluoride ion. Whereas, before an erosive challenge the metal fluoride layer or calcium fluoride layer that forms is highly resistant.

The lack of any significant difference between applying sodium fluoride before and after could be because the application time was too short. Clinically, after fluoride application, the levels of fluoride concentration in the oral cavity remain at potentially active elevated levels for several hours (Duckworth 2013), but in our study they were rinsed off immediately. Also our erosive procedure was relatively long and any effect of the fluoride protection could be removed by the erosive cycling. We used longer exposure times than would be seen clinically with no remineralisation step (Saxegaard & Rölla 1988). This was partially due to the analysis with the profilometer which requires a larger step height difference for more accurate measurements.

This study used pure fluoride solutions and clinically these would not be found in this environment. Although the main active ingredient was fluoride, it would be in combination with other materials either in a tooth paste or mouth rinse and so might affect the action of fluoride to differing levels. There was also no salivary pellicle and this would have had an impact on the efficacy of the fluoride. It would improve the protective effect by its own properties (increased diffusion barrier, high mineral concentration) and acting as a store for the fluoride; increasing in concentration adjacent to the enamel surface and allowing for a longer for diffusion time.

5.7 Summary

The possibility that the different chemistry of stannous and sodium fluoride might have different reactions to the timing of fluoride application could explain some of the conflicting research on erosion. These results showed that under these laboratory conditions, stannous fluoride offered better protection compared to sodium fluoride and that timing of application can be influential. But care must be taken extrapolating these results to the clinical environment as there are several important factors that must be considered. Further, clinical research is needed to establish if there is a relationship between the application of fluoride before or after an erosive challenge. But this investigation also provided the opportunity to use the knowledge gained from earlier investigations in model design.

Chapter 6. Sodium and Stannous fluoride dose response

6.1 Introduction

The aim of this Chapter was to collate the knowledge and techniques gained from the previous Chapters to investigate the effects of a dose-response of the efficacy of sodium and stannous fluoride on enamel erosion. A dose response of sodium fluoride has been shown previously. White et al. investigated, in a laboratory erosion model, the effects of increasing sodium fluoride concentration between 0-450ppm and reported that increasing the concentration increased the protection, but it plateaued at 100ppm (White et al. 2012). Similar fluoride dose studies investigating the response in an erosion model with stannous fluoride have not been reported. The majority of studies investigating stannous fluoride experiments have focused on its effectiveness when combined with other compounds. Investigating both fluorides under exactly the same conditions should allow a direct comparison of the protective effects against erosion and any dose response between sodium and stannous fluoride.

Dose response models are important as they can add to the existing knowledge of a clinically active substance and provide evidence for determining an optimal dose. If there are any negative effects, they can aid in finding a compromise between the desired positive effect and any adverse effects, such as determining the concentration of fluoride to be used in children's toothpaste. They can also be used to create the most cost effective product by allowing for the minimum amount of active substance to be used whilst maintain its efficacy and provide the evidence to produce guidance on use of the active substance.

6.2 Aims, Objective and Hypotheses

6.2.1 Aims

The aims of this laboratory investigation were to investigate the effect of stannous and sodium fluoride in a dose response challenge on enamel following an erosive challenge and assessing changes with step height measurement and Knoop microhardness.

6.2.2 Objectives

- To investigate the protective effect of stannous and sodium fluoride on enamel erosion
- To investigate the dose response of both fluorides at 0, 50, 225, 450 and 1450 ppm

6.2.3 Null Hypotheses

- Increasing concentrations of sodium and stannous fluoride does not affect *in vitro* erosion when applied before an erosive challenge and assessed using step height and Knoop microhardness

6.3 Materials and Methods

Human molar teeth, both caries and crack free, were collected using existing ethical agreements and sectioned at the root using a Buehler Isomet 1000 precision saw with an Extex diamond wafering blade. The samples were mounted in cold cure acrylic resin using a silicone mould, after which they were polished using previously described protocols. After polishing the samples were cleaned with ultrasonication (Nusonics GP-70, T310) by placing them in 80mL of deionised water at 60Hz for 15 minutes. The samples were then rinsed and allowed to dry naturally overnight. A total of 100 buccal surfaces were then randomly allocated to 10 groups.

Once dry, the samples height variation was measured with the profilometer. Any samples that had a variation of greater than ±1.2µm over the test area (approximately 3mm by 1mm) were

rejected. The samples were pre tested for Knoop microhardness with three indentations, 100µm apart, using a dwell time of 10 seconds and a force of 981.2mN. Those samples with a mean outside the range of 330-377H_K were also discarded. Adhesive tape was used to create a window of exposed enamel approximately 1mm by 1mm with reference areas either side. The samples were numbered and stratified randomisation was performed prior to allocating the samples to the appropriate group.

Samples were subjected to a fluoride treatment followed by erosion. For one cycle, samples were first immersed at the determined concentration of sodium or stannous fluoride (0, 50, 225, 450 and 1450), for 1 minute under agitation (Stuart Orbital Shaker SS1, 60rpm) and then were rinsed with 100mL deionised water for 2 minutes under agitation. Afterwards samples were placed in 100mL of static deionised water for 30 minutes, then removed, and placed into 80mL of citric acid solution (0.02M, pH 3.2) under agitation for 10 minutes. They were then rinsed in deionised water for 2 minutes under agitation and the sequence repeated 5 times. For each cycle fresh deionised water and fluoride solutions was used and after the final cycle the samples were allowed to dry naturally in air for at least 24 hours, Figure 41.

6.3.1 Fluoride solutions

50, 225, 450 and 1450ppm solutions of sodium fluoride were made by adding 0.1105, 0.4900, 0.9947 and 3.2053g of Sodium fluoride 99% (Alfa Aesar, lot# 10148378, product code-A13019) to 1 litre of deionised water. 50, 225, 450 and 1450ppm solutions of stannous fluoride were made by adding 0.2066, 0.9300, 1.8592 and 5.9908g of stannous fluoride 99% (Sigma Aldrich, lot# MKBP3104V, product code-100156526) to 1 litre of deionised water. The solutions were mixed with a magnetic stirrer bar until the solid was fully dissolved. The fluoride solutions were used on the day that they were made.

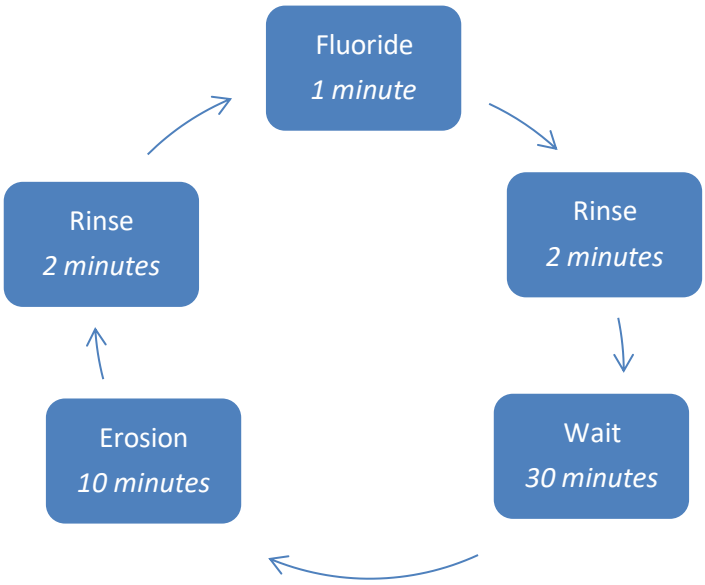

Figure 41 Figure showing the cycling procedure for the fluoride dose-response experiment

6.3.2 Randomisation

The polished and prepared samples were allotted to groups (one control + 2X 4 time points) using stratified random sampling where each group had 10 samples. Each group was numbered and the samples were allotted to each group based on the random allocation.

6.3.3 Measurements

Profilometry and Knoop microhardness measurements were performed for all experiments using the techniques described in Chapter 2.

6.3.4 Sample Size Calculation

As the effect size between sodium and stannous fluoride from the previous experiment (Chapter 5) was high (4.87) the effect size of 1.4 was fixed for the sample size calculation in this study. The sample size calculation for this measure was based on independent samples t test for comparing mean step height/Knoop microhardness between sodium and stannous fluoride separately for each concentration (ppm). Assuming an effect size of 1.4 and 80% power the study required a total sample of 20 (10 per group) to test the significant difference between 2 fluorides at 5% level using a 2 tail test. The power calculation was carried out using gpower3.1.5.

6.4 Statistical Analysis

Descriptive statistics were used to summarise mean step height and Knoop microhardness data for the different groups. The normality assumption for carrying out parametric analysis was tested using Kolmogorov-Smirnov test, Shapiro-Wilk test and histograms. After checking for normality, one way analysis of variance (ANOVA) was carried out to test whether there was any significant difference between groups with respect to MSH and Knoop microhardness. If the ANOVA showed overall significance between groups then further post-hoc analysis using Scheffe's test was carried out to check which two groups differ.

6.5 Results

Figure 42 a and b, show the MSH and KHC with standard deviations for the two fluoride solutions at 0, 50, 225, 450 and 1450ppm. The control group produced a MSH of 11.0µm (±1.1) and a KHC of 151.0H_K (±30.4). For both sodium and stannous fluoride, as the concentration of fluoride increased there was a corresponding decrease in the mean step height. For sodium fluoride, the largest mean step height was 9.7µm (±1.2) at 50ppm and the lowest was 6.3µm (±0.6) at 1450ppm. At comparable concentrations, stannous fluoride produced statistically lower mean step height compared to sodium fluoride ($p<0.001$). For stannous fluoride, the largest mean step height was 6.9µm (±0.7) at 50ppm and the lowest was 3.7µm (±0.5) for 1450ppm.

The Knoop microhardness change showed no clear patterns with increasing the concentration of sodium and stannous fluoride. All groups produced a significantly larger change in the Knoop microhardness compared to the control 151H_K (±30.4) ($p<0.05$). Stannous fluoride produced a larger Knoop microhardness change of 192.4H_K (±18.9), 194.6H_K (±25.2), 160.6H_K (±21.3) and 174.7H_K (±25.8) at 50, 225, 450 and 1450ppm respectively compared to sodium fluoride 156.2H_K (±28.0), 176.6H_K (±25.2), 147.4H_K (±21.3) and 155.1H_K (±25.4) for 50, 225, 450 and 1450ppm respectively.

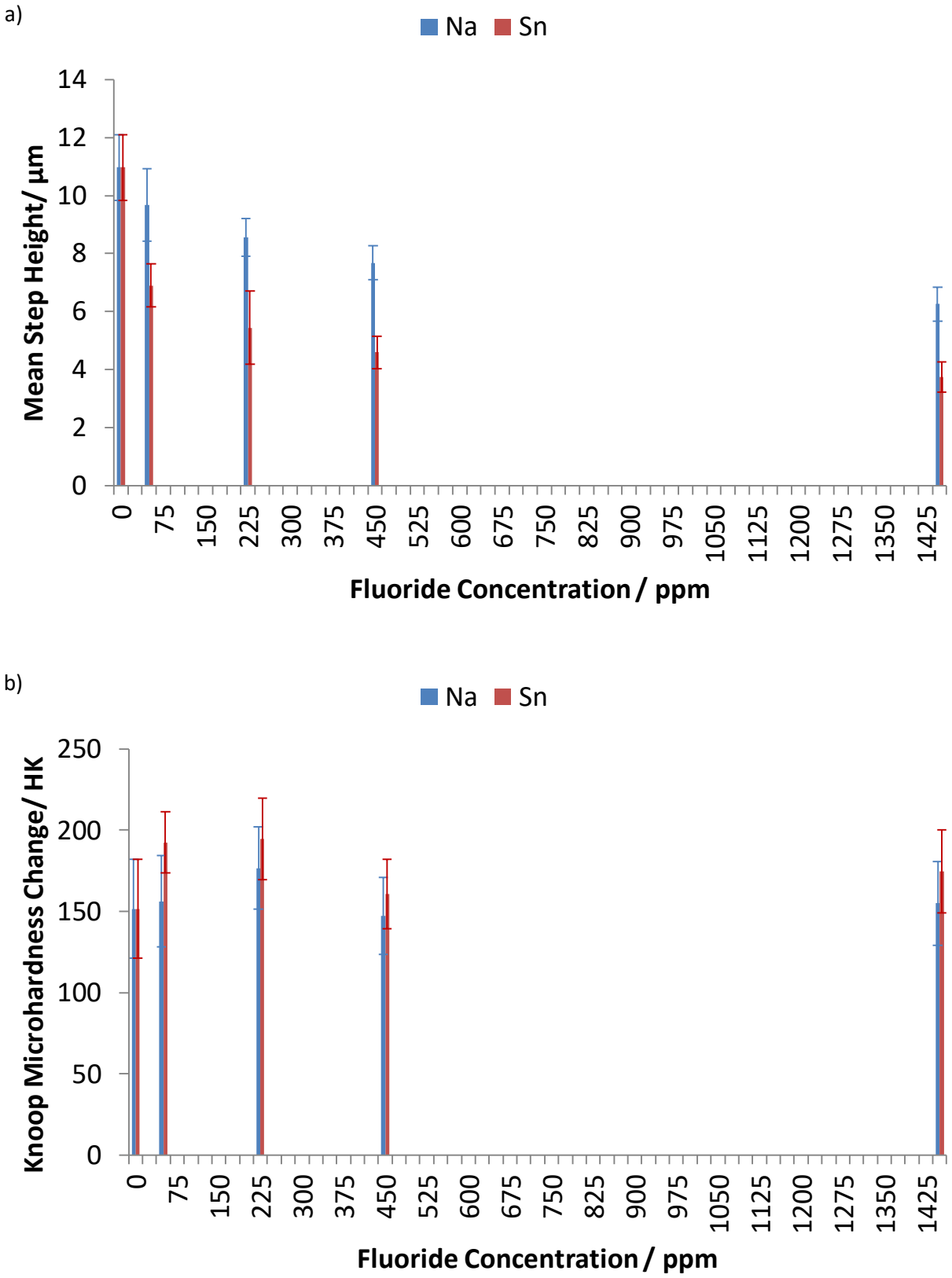

Figure 42 a) Mean step height (µm) and b) Knoop microhardness change (H_K) with standard deviations at 0, 50, 225, 450 and 1450ppm as indicated for the sodium and stannous fluoride dose response experiment. Blue lines represent sodium fluoride and red lines represent stannous fluoride

Table 9 shows a homogeneous groups table for the mean step height data. The start and end of a line represents a significant difference ($p<0.05$) between the groups. For example, the line furthest to the left indicated a significant difference between the control (0ppm) and sodium fluoride 225ppm. All groups showed a significant difference compared to the control except for sodium fluoride at 50ppm. There is no table for the Knoop microhardness data as none of the groups showed a significant difference.

Dose	Mean/μm (±SD)
NaFl	
0	11.0 (1.1)
50	9.7 (1.2)
225	8.6 (0.6)
450	7.7 (0.6)
1450	6.3 (0.6)
SnFl	
50	6.9 (0.7)
225	5.4 (1.3)
450	4.6 (0.6)
1450	3.7 (0.5)

Table 9 Homogeneous groups table showing statistical differences ($p<0.05$) between each sodium and stannous fluoride concentration for mean step height data. significant difference

6.6 Discussion

This dose response experiment was performed to expand the results from Chapter 5. The change to the sample preparation produced closer baseline microhardness and surface flatness ranges, combined with strict randomisation, ensured a greater standardisation between the groups. The fluoride concentrations were chosen to represent clinically applicable levels found in toothpastes and mouth rinses. The results showed that compared to a deionised water control, both sodium and stannous fluoride produced statistically significant protection of enamel in a citric acid erosion model, except for sodium fluoride at 50ppm. The samples in the control group had no effect from fluoride and so the addition of fluoride to the experimental groups and their resulting lower MSH, suggest that the metal fluoride ions provide erosive protection. With increasing concentration of fluoride a decreased mean step height occurred and suggested that greater protection was offered at higher concentrations. Also, the results showed that under these experimental conditions, stannous fluoride provided better protection than sodium fluoride. The step height data confirmed that increasing the concentration of fluoride increased the protective effect against erosion.

The results for Knoop microhardness data did not show a dose response effect. Compared to the control, the data showed a greater change and more surface softening for stannous fluoride than sodium.

The concentrations of fluoride investigated were selected to allow for a dose response effect that was clinically applicable. The concentrations in mouthwashes (225ppm) and toothpastes (1450ppm) were selected and used to compare to the lower concentrations to assess the dose response over a wide range. The range is typical of other dose response type experiments (Gracia et al. 2010; White et al. 2012; Kato et al. 2014).

This model used a modified sample preparation compared to the previous experiments in this thesis. The main area of change was quality control. For this study, a tighter baseline

microhardness value was used (between 330-377H_K) to select samples and followed other authors (Manarelli et al. 2011). However there is no range that is widely accepted by most researchers. Another area was the randomisation technique, using tighter baseline microhardness values, the samples were randomised using stratified randomisation so that each group would have an equal distribution of Knoop microhardness. The randomisation reduced the possibility of bias whereas the quality control ensured samples were as similar as possible.

The decrease in mean step height with increasing the concentration of fluoride reflects the increase in availability of fluoride and metal ions in solution. A higher fluoride ion concentration means greater amounts of fluoride being either deposited onto or incorporated into the enamel surface. For stannous fluoride, the higher concentration could result in a quicker forming or a thicker metal-fluoride layer. Ganss et al. suggested that the sodium ion has a role in the dissolution of a CaF_2-like layer, which would explain the lower efficacy of sodium fluoride compared to stannous fluoride (Ganss et al. 2008).

At the same ppm (e.g. sodium fluoride 225ppm and stannous fluoride 225ppm) the concentration of fluoride in the solution is the same, so the difference in protection between them was an effect of the action of the metal ions. It is difficult to say conclusively whether the higher concentration of stannous fluoride would result in a quicker or thicker metal fluoride layer, if it was present, based on these findings. It could be that at the higher concentrations the metal fluoride layer was both thicker and faster forming. These theories could be tested by using a wider range of analytical tools and this is discussed further in Chapter 7 in the future work section.

The only clear patterns from the Knoop microhardness data is that all groups showed a greater softening compared to the control and that the stannous fluoride group produced softer surfaces compared to sodium fluoride. It can also be seen that for both fluorides the 50 and

225ppm produced softer surfaces compared to the 450 and 1450 groups. This could reflect that the higher concentrations had sufficient ions in the solution to form the harder fluorapatite in greater proportion compared to the lower concentrations. This finding that stannous fluoride produced a softer surface but less tissue loss is consistent with the results from the previous Chapters and suggests that both fluoride ions work in different ways to resist the effects of acids. The microhardness data could also reflect the finding that the Knoop indenter or these setting were not sensitive enough. The indenter might have penetrated any protective layer on the surface and through any demineralised softened enamel. This would then be measuring the hardness of underlying harder enamel. This could explain the data as there was a significant difference in the mean step height between the fluorides yet there appears to be no significant difference for the microhardness.

White et al. investigated the effects of sodium fluoride protection at 0, 1, 10, 50, 225 and 450ppm on hydroxyapatite dissolution with and without a salivary pellicle (White et al. 2012). Their study was relatively comparable as they reported the results from 50, 225 and 450ppm of sodium fluoride, without a pellicle and using citric acid as the erosive agent. However, the results cannot directly be compared as they used hydroxyapatite dissolution compared to step height and microhardness as the measure of erosion; 1.0% citric acid (pH 3.75) with 2 minutes exposure time compared to 0.02M (0.3%) citric acid (pH 3.2) with 10 minutes exposure time; 30 seconds of a single fluoride exposure compared to 1 minute of multiple fluoride exposures and there was no stannous fluoride investigated. Nevertheless, they reported that at 10ppm sodium fluoride provided some protection against erosion and a dose response was observed with an upper limit at approximately 112ppm. Our results also showed a dose response for both fluorides measured as mean step height, however, a plateau was not observed. Perhaps higher concentrations and smaller increments of fluoride concentrations might produce a similar plateau so further work is needed but this would become less clinically relevant.

A similar study by Gracia et al. investigated the protective effects of 0, 100, 112, 225 and 450ppm sodium fluoride solutions against *in vitro* citric acid erosion (Gracia et al. 2010). They reported that increasing concentration of fluoride from 100, to 112 to 450 produced a decrease in the mean step height with 1.23μm (±0.41) to 0.48μm (±0.48) to 0.18μm (±0.13) respectively which shows a similar trend to this chapter. The results in this chapter produced larger mean step height, 7.7μm (±0.6) at 450ppm compared to 0.18μm (±0.13). This difference could be down to several possibilities, the experiment in this chapter used a longer immersion time (50 minutes compared to 5 minutes) with a lower pH acid (3.2 compared 3.8). It could also be explained by the agitation of the solution, described in Chapter 4, and this is supported by other authors (Shellis et al. 2005; Attin et al. 2012).

A study by Kato et al. investigated the effects of different concentrations of sodium fluoride on its ability to inhibit matrix metalloproteinases (MMP) (Kato et al. 2014). Whilst this is not directly comparable to the results from this study the implications of the work are fascinating. In extreme cases of erosion, dentine will be exposed. Acid attack on dentine dissolves mineral component leaving a demineralised organic matrix (DOM). The DOM might act as a barrier to further demineralisation and therefore tissue loss. However, the presence of MMP's could degrade the DOM. Kato et al. investigated 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 500, 1500 and 5000ppm sodium fluoride solutions and reported that sodium fluoride completely inhibited the activity of MMP's from 200ppm onwards with decreased activity below this concentration. This shows that fluoride concentrations used in mouth rinses and toothpastes could be beneficial for erosion prevention even after sever erosion to the point where dentine is exposed.

These results shown here also confirm the data from Chapter 5. Chapter 5 showed that stannous fluoride provided better protection compared to sodium fluoride. This experiment, investigated the effect of fluoride application before an erosive challenge, which replicates

what would most likely happen clinically however, as the work in Chapter 5 investigated, applying after an erosive challenge might also have an impact on the efficacy of the solution. It would be interesting to apply the same principle to this experiment. As with all the experiments in this thesis, a salivary pellicle was not utilised and so these results must be carefully interpreted when applying to the clinical environment.

The more stringent sample preparation and quality control benefitted the study outcome. The standard deviations were smaller and appeared to be closer between the groups. Even tighter baseline Knoop microhardness and surface height variation could have been employed however a balance must be struck between the ideal situation and what is practically possible. A study into the effects of different baseline values on the measurement outcome would be beneficial to *in vitro* research as it would give the researcher more confidence in selecting appropriate baseline values that would be achievable without having a negative effect on the study outcome. Other developments of the model also will have influenced the results. Previous chapters have reported changes with; the selected tooth surface to erode, ultra-sonicating the samples after polishing, the immersion protocol and stirring. These results combine all the experiences developed during the study to create a more controlled laboratory condition. This was reflected in the smaller standard deviations.

Many of the points mentioned in Chapter 5 apply here also. These experiments do not replicate the clinical environment (although no current experimental set up could) and the fluoride solution differs from commercial mouth rinses/toothpastes which contain many other ingredients. No salivary pellicle was used here and this might have influenced the data and the outcome although this illustrated the effect of fluoride directly onto eroded enamel.

Dose response experiments tend to focus on sodium fluoride however the stannous fluoride results presented here show that this can be applied to other fluorides. Further work should

be undertaken to investigate the dose response effect of the other fluorides that are available on the market as this would be beneficial to industry and the patient.

6.7 Summary

This experiment showed that under these conditions, increasing the fluoride concentration provided better protection against *in vitro* erosion and reject the null hypothesis. It also showed that stannous fluoride offers better protection than sodium fluoride when measuring the mean step height. Knoop microhardness does not show any clear patterns when increasing the concentration however it can be seen that stannous fluoride produced a softer surface compared to sodium.

Chapter 7. General summary and conclusions; suggestions for future work

7.1 General summary

Introduction

A recent paper titled 'Methodology and models in erosion research' summarised discussions from a workshop on methodology used in erosion research. The authors suggested that it was essential that experimental conditions should be controlled and reported in detail; such as the erosive agent, pH, method of agitation, consistency of composition, temperature and duration of erosive challenge (Shellis et al. 2011). In 2013, researchers in the tooth erosion field still have difficulty in accurately interpreting results as there are no universally accepted protocols for *in vitro* studies (Ganss et al. 2013), this, along with the reporting was addressed in a concept note in 2014 that suggested guidelines to improve the current situation (Krithikadatta et al. 2014). These suggestions were the stimulus for this PhD but also a need within the laboratory to improve the data collection and accuracy of the work. Following this PhD significant changes to the laboratory models have occurred and two papers have been published outlining these.

Measurement techniques

Profilometry and Knoop microhardness were the main methods used in this thesis to measure changes in the enamel surface. Knoop microhardness produced variable results with higher standard deviations even after controls were in place to standardise the enamel surfaces prior to the laboratory investigations. The basic microhardness indenter is designed for metals, not enamel, which although it is a rigid organic substance it behaves differently to metals under deformations. To accurately record microhardness the surface needs to be flat and reflective, which is possible with highly polished enamel. However, following erosion the enamel surface

changes, increasing the difficulty to visualise the indenter's shape and therefore increases the difficulty for measurement. The conflict for early erosion lesions is that profilometry is also challenging as the step heights produced are at the extreme of the systems resolution. The baseline criterion for experiments in Chapter 4 and 5 were 340 ±50H_K, which was a lower H_K, and therefore a softer surface with a larger range. On reflection these parameters were too large and so for Chapter 6 the range was reduced to 330-377 H_K. There is little, if any, guidance from the literature as different research groups use different thresholds. This smaller range provided a more uniform baseline between the samples and groups. Ideally a much tighter range for the baseline would have been used in the earlier Chapters. However, if a tighter criterion were employed, adequate sample attainment would have been difficult in the time frame.

Profilometry was used as the gold standard for the measurement of surface change on enamel. The data output was step height change but other surface characteristics, such as roughness, could also be used for future investigations. The major challenge of profilometry was that a flat surface was required to measure the step height change. A flatter surface allows for smaller changes to be accurately observed but as mentioned in Chapter 3 the limits of our polishing protocol meant that step height change values of less than 2µm were difficult to measure accurately. This resolution for an early-erosion lesion would have been inappropriate but the magnitudes of step height measured in this thesis were sufficient. The standard erosive protocol (for Chapters 3, 4, 5 and 6) was designed to produce a measurable step height (accounting for the sample flatness and the non-contact white light profilometers specification), which was greater than 2µm.

Chapter 3

Chapter 3 showed that erosion-only was predictable and uniform in the *in vitro* model but the addition of abrasion was more complicated. For example, the data from citric acid, at 10

minutes immersion, increasing the concentration from 0.02 to 0.03 to 0.05M produced an increased mean step height of 7.9µm (±1.1), 14.2µm (±2.8) and 18.4µm (±3.0) respectively. This increase in concentration and immersion time relating to an increase in MSH was also observed for citric and phosphoric acid. The effects of the different acids and concentrations have been shown before but not specifically with citric, phosphoric and hydrochloric acid under the same conditions of pH and erosive cycling.

The data from the hydrochloric acid showed that the concentration was altered too much that it became ineffective in causing erosion. Whilst this allowed for a comparison between all the different acids it did not represent the clinical situation and future work with these acids should be performed at their native pH.

The results from the abrasion showed that further work is needed to investigate why the relationship with erosion was not uniform. Modelling abrasion *in vitro* is difficult due to the complex clinical situation and as a result the model needed to be simplified and, this could be a reason why the relationship was not uniform.

Chapter 4

Chapter 4 investigated the effect of variables in a laboratory model on erosion and showed these had a significant effect on the measurable outcome on enamel. This showed small changes to the method from sample preparation to experimental procedure, impacted upon the outcome. For example, for 'storage of samples' and 'rinsing between cycles' the main finding was longer storage in deionised water or different types/ lengths of rinsing had no significant effect, however, there was a significant effect observed for storing dry or not rinsing. However, all these findings highlight the importance of accurate and detailed reporting and consistency in performing the experiment. Even after investigating these variables there many more that could be assessed. An example would be the tooth surface and type. Although

bovine enamel has been studied by other authors, it would be interesting to investigate how it would behave in this experimental model. Furthermore, only the buccal and lingual surfaces of molar and premolar teeth were compared but the effect of anterior teeth might also be interesting to assess.

Chapter 5

The fluoride work in Chapter 5 provided evidence on how the application of fluoride, before or after an erosive challenge changed the level of erosion. Previous research on different fluorides suggested that stannous fluoride had better profound erosive protection. The results from this Chapter showed a significant difference between stannous fluoride with a MSH of 14.0µm (±2.0) and sodium fluoride with a MSH of 24.9µm (±2.3). Stannous fluoride also showed a significant difference when applied after erosion (7.5µm ±0.8) than before (6.5µm ±1.2) and showed that the efficacy of stannous fluoride differs suggesting it has a greater impact on preventing demineralisation. Recently, fluorides have been combined with other compounds to improve the efficacy of oral healthcare products as seen by the dual phase calcium silicate/phosphate gel combined with a calcium silicate/ phosphate tooth paste (Joiner et al. 2014). Exploration of this area could open up more possibilities for combinations of fluorides and gel/varnish systems, making the need for solid laboratory testing even greater.

Chapter 6

Chapter 6 investigated a dose response for sodium and stannous fluoride at 0, 50, 225, 450 and 1450ppm concentrations. The experiment was designed following the results from previous chapters on model development Further changes to the sample preparation and randomisation were employed with tighter control of the Knoop microhardness values at baseline and this provided data with lower standard deviations. This showed a dose response effect for sodium and stannous fluoride with significant differences between them with the

step height ($p<0.05$) however no dose response was seen for the microhardness data. Further work is needed by assessing a greater range of concentrations at regular intervals to determine at which point a plateau effect is observed and to understand the relationship between the fluoride and its effect more accurately. This work does not address the mode of action of the fluoride and what effect the concentration has on the proposed mechanisms; this can be explored in future work using a greater variety of measurement techniques.

Overall summary

A reliable, accurate, clinically relevant *in vitro* model to study tooth erosion would be of great benefit. Testing new formulations would become easier, cheaper and comparable between different research groups. As the knowledge in the area of tooth erosion improves and technology changes, the aim would be to create an *in vitro* model that would accurately represent the clinical situation; reducing the reliance for expensive, time consuming *in situ* models, however this ideal may be impossible, due to the complexity of the oral cavity. However, as our understanding of the role of saliva and fluid dynamics within the oral cavity improve these can be added to the *in vitro* models, helping them to provide results that are more representative of the clinical situation.

A universal *in vitro* erosion model might be possible for product testing. If such a model existed it would potentially mean, if correctly implemented, all the research could be compared. This could be achieved by creating agreed standards, similar with the American Society of the International Association for Testing and Materials (ASTM) or the International Organisation for Standardisation (ISO). This would be particularly useful for testing the anti-erosive effects of a product, as it would allow the claims to be comparable.

Whilst there will inevitably be variation in study designs, better and more accurate reporting of the study conditions would allow for improved understanding. This challenge lies mainly with

the journal editor and authors to enforce the correct reporting of *in vitro* studies. This would require a similar set of guidelines such as the Consolidated Standards of Reporting Trials (CONSORT) to be applied and enforced to *in vitro* studies. Krithikadatta et al. suggested a concept, called the Checklist for reporting In-vitro Studies (CRIS) (Krithikadatta et al. 2014). The concept sets out a guideline for reporting sample size calculation, detailed sample preparation and handing, randomisation and blinding and the statistical analysis, which would increase the quality and transparency of evidence. This would also impact on systematic reviews and meta-analysis of *in vitro* studies becoming more comprehensive and useful.

7.2 Conclusions

Within the limitation of these *in vitro* studies, the following conclusions were made:

- The three techniques to mathematically calculate the step height showed a high level of agreement with an interclass correlation of 88%. This showed that there was little difference between the methods; however, MountainsMap® was selected for all analysis as it used it used the ISO 5436-1 standard
- Generally, increasing the immersion time and concentration, increased the mean step height and therefore erosion for citric and phosphoric acid but not for hydrochloric acid
- The addition of abrasion after erosion did not uniformly increase the mean step height
- There was no significant difference in the mean step height between buccal and lingual surfaces on molar and premolar teeth ($p=0.152$). Molar lingual surfaces produced significantly higher Knoop microhardness change ($p<0.05$) compared to molar buccal surfaces
- Ultrasonicating the samples produced significantly lower ($p<0.05$) mean step height and Knoop microhardness change
- Storing samples wet compared to dry produced significantly lower mean step height and Knoop microhardness change ($p=0.049$ and $p<0.05$) however there was no significant difference between increasing the storage time from 1 to 24 hours
- Increasing the speed of agitation increased the mean step height for all agitation types. See-saw agitation produced the greatest mean step height followed by Gyro and Orbital at 30rpm. There was a significant difference ($p<0.001$) in the mean step height and Knoop microhardness change between the three types of agitation compared to the control

- The effect of not rinsing produced a significantly lower mean step height and Knoop microhardness change ($p<0.05$) compared to rinsing however there was no significant difference between the different types or length of rinsing
- Increasing the volume of the erosive solution from 80 to 100mL did not increase the mean step height and Knoop microhardness change
- Positioning the sample so that the enamel surface faces 'downwards' into the solutions produced significantly less mean step height and Knoop microhardness change ($p<0.05$) than with the sample facing 'up'
- At low ppm concentration, stannous fluoride provided better protection against erosion than sodium fluoride solution as it produced significantly lower mean step height ($p<0.05$)
- Applying stannous fluoride before an erosive challenge was more effective as it produced a significantly lower mean step height ($p<0.05$) compared to application after an erosive challenge
- A dose response effect was seen for sodium and stannous fluoride with stannous fluoride providing significantly greater protection in terms of mean step height ($p<0.001$) and softer surfaces with respect to Knoop microhardness ($p<0.05$) compared to the control

7.3 Future Developments

- To make the current *in vitro* erosion model even more clinically relevant by accurately replicating the conditions found in the oral cavity, such as:

Temperature - to make the temperature changes closer to the clinical situation. Incorporating the; stirring, addition and removal of samples by using a water bath, or heating element. Exploring the effect of adjusting the temperature of a solution before exposure to enamel samples.

Salivary pellicle - either a natural or artificial pellicle would influence the erosion model and make it more clinically relevant. For example, using fresh v frozen might affect the outcome.

Tongue/check abrasion - exploring materials and application methods that might mimic the action of the tongue and cheek more accurately.

Fluid motion - studying the fluid dynamics that might occur in the oral cavity and applying them to an *in vitro* model by developing specialised stirrers and solution (acid or saliva, or mouth rinse) addition and removal systems.

To investigate more of the *in vitro* erosion model variables, such as: effect of polishing (final grit size, smear layer thickness, constituents dependant on polishing materials). Investigating the effect of using enamel surfaces polished to FEPA grit sizes 600, 1800 and 2400, might have different outcomes. A study into the smear layer created after polishing samples using silicon carbide, aluminium oxide or silica oxide based grinding/polishing materials could be undertaken with SEM, SEM-EDX or ICP-MS.

Position of the sample within the container (e.g. centre or corners) -investigating whether the position of the sample in a solution when faced up, has any impact on the amount of erosion caused and this might be linked to fluid motion.

To use a wider range of instruments such as inductively coupled plasma mass spectrometry, Optical Coherence Tomography, pH stat and Scanning Electron Microscopy to further investigate *in vitro* erosion using the model. Using a wider selection of analytical methods in the *in vitro* model to gain a greater understanding of effects of erosion and to integrate the findings with the profilometry and microhardness data. To investigate the effects of fluoride application before or after and the dose response in greater detail by; assessing a larger variety of fluorides, concentrations, commercial products.

- To study the mode of action of the different fluorides.

The dose response study presented in Chapter 6 could be expanded by investigating a larger range of doses, and at regular intervals might allow for a better comparison of the relationship between the fluoride and its' effect on erosion. As no plateau effect was observed this could be explored by investigating concentrations below and above the range investigated in this thesis. Investigating low concentrations such as 0, 5, 10, 15, 20, 25, 30, 35, 40 and 45ppm might show the concentration of fluoride at which it begins to be effective. At the higher concentrations, investigating; 1500, 2500, 3500, 4500, 5500… to 12500 might show if or at what level a plateau effect was reached.

Another area to be investigated could be to study the formation and thickness of the CaF_2 protective layer produced by the different metal fluorides. This could be explored with imagining techniques, such as transverse microradiography, which might reveal if stannous

fluoride application creates more surface softening. The thickness of this layer could also be investigated by transverse microradiography but also scanning electron microscopy and optical coherence tomography. SEM might characterise the surface layer after exposure and OCT might show the speed of formation. Nanoindentation could also be used to provide more accurate and sensitive hardness data that could probe the properties of the CaF_2 layer and also the eroded enamel surface.

The effect of the metal ion incorporating into the enamel surface can be investigated by SEM-EDX or Secondary ion mass spectrometry. SEM-EDX will reveal the presence of ions at the surface of the enamel. Secondary ion mass spectrometry reveal if and how far into the enamel surface the fluoride metal ions penetrate and also the presence of any other elements.

Chapter 8. References

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Chapter 9. Appendices

9.1 Patient Information Sheet

Guy's, King's and St Thomas' Dental Institute
Department of Fixed and Removable Prosthodontics
25th Floor, Guy's Tower
Guy's Hospital
London Bridge
London
SE1 9RT
Tel 0207 188 5390
Fax 0207 188 7486

Volunteer information sheet (Version 2) 13/11/12
Title of project: Protection of erosive tooth wear (donation of extracted tooth)
REC ref: 12/LO/1836
Investigator: Professor David Bartlett

You will be given a copy of the information sheet and a signed consent form to keep.

Part 1
Invitation paragraph

You are being invited to donate your tooth for a research study. Before you decide it is important for you to understand why the research is being done and what it will involve:

Part 1 tells you the purpose of the study and what will happen if you decide to participate.
Part 2 gives you more detailed information about the conduct of the study.

Please take time to read the following information carefully. Ask us if there is anything that is not clear. Talk to others about the research if you wish and the following organization could give you independent advice:
Guy's and St Thomas' Hospital NHS Foundation Trust Patient Advice and Liaison Service Telephone 020 7188 8801 or 020 7188 8803 email: pals@gstt.nhs.uk
Post: Patient information team, Knowledge and information centre, St Thomas' Hospital London, Westminster Bridge Road, SE1 7EH

What is the purpose of the study?
Tooth wear is a condition where the teeth wear away faster than normal and is caused by acid erosion (from acidic foods and drinks and stomach acid), tooth grinding and over brushing. Tooth wear is a common condition that can affect anyone and it appears to be happening more and more nowadays. Severe tooth wear can cause teeth to become very sensitive, as well as causing cosmetic and chewing problems due to shortened teeth and even in severe cases can cause tooth loss. Certain toothpastes and mouth rinses have the potential to prevent and treat tooth wear. However the scientific evidence for this is lacking. This study will us help to identify products that can be beneficial in preventing and treating tooth wear and improve the advice that we can give Volunteers on preventing tooth wear.

Why have I been chosen?
You are suitable for this study because you are a healthy individual who needs a tooth removed.

Do I have to take part?
It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw

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at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I decide to take part?
At your first visit, when you are consulted about the tooth extraction, you will be invited to join the study by a clinician. At your second visit we will confirm that you still want to donate your tooth and then you will have your tooth removed in the normal way. After your tooth is extracted it will be transferred to the Biomaterials laboratory at King's College Hospital Dental Institute (Department of Biomaterials, 17th Floor, Guy's Tower, Guy's Hospital, London Bridge SE1 9RT). Once the tooth is extracted your participation in the study is over.

What do I have to do?
You will just have to attend your set appointments as normal.

What is the drug, device or procedure being tested?
Various dietary acids, fluorides and other protective agents are being investigated in this study on the extracted teeth.

What are the alternatives for diagnosis or treatment?
The research does not involve any Volunteer treatment and you will receive your routine standard treatment as usual.

What are the side effects of any treatment received when taking part?
There are no risks associated with this study, other than the usual risks of a tooth extraction which will be explained to you by the clinical team who are carrying out the treatment.

What are the other possible disadvantages or risks of taking part?
There are no risks associated with this study, other than the usual risks of a tooth extraction which will be explained to you by the clinical team who are carrying out the treatment.

What are the possible benefits of taking part?
We do not expect that you will receive any benefit from taking part in this study.

What happens when the research study stops?
We aim to publish the results in medical journals.

What if there is a problem? And contact details:
No problems can be foreseen however the contact number for complaints or concerns is for: Professor David Bartlett 0207 188 5390 or email david.bartlett@kcl.ac.uk

Will my taking part in the study be kept confidential?
We will not be collecting any information about you and your confidentiality is safeguarded during and after the study. Our procedures for handling, processing, storage and destruction of your data are compliant with the Data Protection Act 1998.

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Contact for further information:
Professor David Bartlett 0207 188 5390 or email david.bartlett@kcl.ac.uk

This completes Part 1 of the Information Sheet. If the information sheet in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

Part 2

What if relevant new information becomes available?
We are a leading establishment in this area of research and if any new information relevant to this study becomes available the researchers will discuss this with you. You are free to withdraw from the study at any time.

What will happen if I don't want to carry on with the study?
You can withdraw from the study. Just advise the clinician treating you that you do not want to donate your tooth and your tooth will be disposed of once extracted, or you can keep it to take home.

What if there is a problem?
If you have any concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer their questions.
Professor David Bartlett 0207 188 5390 or email david.bartlett@kcl.ac.uk

If you remain unhappy and wish to complain formally, you can do this through the NHS complaints procedure. If you are harmed by taking part in this research project there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay privately for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way that you have been approached or treated during the course of this study, the normal NHS complaints mechanisms should be available to you.

Details of how to complain can be obtained from the Volunteer Advice and Liaison Service (PALS)
Guy's and St Thomas' Hospital NHS Foundation Trust Patient Advice and Liaison Service
Telephone 020 7188 8801 or 020 7188 8803 email: pals@gstt.nhs.uk
Post: Patient information team, Knowledge and information centre, St Thomas' Hospital London, Westminster Bridge Road, SE1 7EH

Will my taking part in this study be kept confidential?
We will not be collecting any information about you and your confidentiality is safeguarded during and after the study. Our procedures for handling, processing, storage and destruction of your data are compliant with the Data Protection Act 1998.

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What will happen to any samples that I give?
After your tooth has been removed, it will be anonymised (i.e. there will be no way of linking the tooth to your personal data or medical records) and then transported to the Biomaterials laboratory at King's College Hospital Dental Institute (Department of Biomaterials, 17th Floor, Guy's Tower, Guy's Hospital, London Bridge SE1 9RT). The tooth will be used in a laboratory study investigating the effects of acid on tooth wear and the ability of topical protection in preventing and treating tooth wear. The study is a laboratory experiment which involves simulating tooth brushing and acid erosion on the tooth in the laboratory, as well as exposure to topical protection. Following this, measurements of the amount of wear on the tooth surface are taken.
Once the study is completed the tooth samples will be carefully disposed of in accordance with local and national guidelines.

What will happen to the results of the research study?
The results of the study will be published in medical journals. Participants will not be identified in any report or publication.

Who has reviewed the study?
This study was given a favourable ethical opinion for conduct by NRES committee London-Bloomsbury.

Will any genetic tests be done?
No.

Thank you for considering taking part and for taking time to read this sheet – please ask any questions if you need to.

9.2 Patient Consent Form

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25th Floor, Guy's Tower
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London Bridge
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Tel 0207 188 5390
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Consent Form (Version 1) 30/10/12
Title of project: Protection of erosive tooth wear (donation of extracted tooth)
REC ref: 12/LO/1836
Investigator: Professor David Bartlett

Please complete this form after you have read the Information Sheet and/or listened to an explanation about the research

Patient Identification: Date

Thank you for considering taking part in this research. The person organising the research and/or a member of the clinical team who is trained for this purpose must explain the project before you agree to take part.

If you have any questions arising from the Information Sheet or explanation given to you, please ask the researcher before you decide whether or not to join in. You will be given a copy of this Consent Form to keep and refer to at any time.

I confirm that I have read and understand the information sheet dated (version 2) for the above study.

I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.

I agree to take part in the above study.

Name of Patient.....
Signature.....Date.....

Name of Person taking consent.....
Signature.....Date.....

1 for patient, 1 for researcher site file, 1 (original) to be kept in medical notes, 1 to be kept with donated tooth
Page **1** of **1**

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